

Cutting off the sap to US research

Cutting back on government by the Republican Congress threatens to damage one of the few things US governments have consistently done well in the past half century — providing public support for research.

REPUBLICAN control of both houses of the US legislature is beginning to hurt, both directly, in the scamp for budget cuts, and indirectly, by inducing the White House to anticipate what the Republicans are likely to accomplish. All kinds of constituencies are complaining; moves to cap the growth of Medicaid have the army of the retired up in arms, those who depend on Social Security are wondering when they will be impelled towards the battle lines. With great social changes of that magnitude in the offing, special pleading on behalf of minor interests is unfashionable. But have the Republicans thought through the likely consequences of their zeal to reduce the role of government for the US government's outstanding achievement since the Second World War: the success of the US research enterprise?

Since Vannevar Bush published his *The Endless Frontier* in 1947, there has been broad and bi-partisan support in the United States for the steady strengthening of the research enterprise. At the beginning, that was easily won. Had not the Second World War been brought to a conclusion by the efforts of researchers, not only at Los Alamos, given unstinted backing by a hopeful government? Might not a continuation of that backing help the United States to win the peace as well as the war? Until quite recently, the expectation seemed to have been fulfilled. The post-war resilience of the US economy and its capacity for technical innovation provided the economic resources for the regeneration and the defence of much of Western Europe, but also provided the US population with unparalleled prosperity. Are those not the benefits of Vannevar Bush's recipe?

Proof

Proof is hard to come by. Academics are among the first to point to the difficulties of relating public spending on research to industrial innovations that sweep the world. Certainly it would be impossible to tell in retrospect which (if any) research grant gave the world the photocopier (a product of the Xerox Corporation) or made the Boeing Corporation into the world's most successful aircraft manufacturer. Intel's success in the design and manufacture of microchips is similarly obscure, perhaps because it is in part a product of the Pentagon's fancy for integrated circuits in the 1970s. The antecedents in research laboratories of the biomedical innovations occasionally mistaken for signs that immortality is at hand in the United States are more easily traced, but even there the link is far from clear. With the

benefit of hindsight, competent professional managers of research would easily devise ways in which the present crop of innovations could have been brought to their present conditions more cheaply than by giving the National Institutes of Health (NIH) \$10 billion a year to spend.

Skills

What experience has really shown is that these sceptical arguments are irrelevant to the question of the proper relationship between a government and its research enterprise. The necessary and sufficient condition for the public support of research is not innovation as such, but the creation and maintenance of a pool of technical skill. That is as true of the United States as of its smaller economic competitors. For the time being, high-energy physics may be irrelevant to the prosperity of the United States, but US industry has a great need of the skills in data-gathering and handling or in precision-engineering design that professionals in the field have and, at the worst, can teach to others. And who will say that skill in wringing a few extra photons-worth of data from a telescope is irrelevant to the strategic and economic needs of the United States?

Innovation is not unimportant, of course. And the US research enterprise, even at the academic end of the spectrum, has been prolific. Perhaps between two-thirds and three-quarters of the world's excellent research in quickly growing fields is now the product of US laboratories. And much of that is immediate grist to the mill of the host of companies that now follow the literature of science with the legendary zeal of newspaper reporters following fire engines through the streets of major cities in the hunt for calamity. The United States is better supplied with mechanisms for the commercialization of the results of its research than any of its competitors. So why should the US Congress be mean with money for research?

Cutting back on the deficit is a worthy cause, of course. And research spending is in the vulnerable category of discretionary spending. But it is mistaken to suggest, as many Republicans do, that the system of research support can only be made more efficient if the competition for research funds among researchers is made even more intense. Already there are signs that competitiveness has gone too far in the hunt for grants. Why else the rash of charges of misconduct a year ago, and the persistent tendency to publish accounts of even successful research programmes in fragments? But the real victims of the present system are



the talented people thrown out of academic laboratories indecently soon after some committee has turned down their application and the design of the research people say in their applications they will attempt, in which caution takes precedence over imaginativeness.

What the US research enterprise really needs is not a tighter competition for research funds, but a looser one. A shift (modest to begin with) towards a system of support for laboratories and institutions (for which laboratories and institutions would compete among themselves) would help to make the US research enterprise more durable and more productive. Nobody could guarantee that the result would save money for the Congress, but it need not cost more. To the extent that the unrecognized victims of present competitiveness include neglected students, the benefits of change for the enlargement of the stock of skill in the United States could be substantial.

The recommendation that the intramural research programme of the National Cancer Institute should be trimmed back (see page 267) is a different kettle of fish. For many years, it has been anomalous that the intramural programmes at the National Institutes of Health (NIH) as a whole are not objectively evaluated by others than themselves. It can only be in the US public's interest that this should now be done; the cancer institute is simply the first in line. But again, the solution is not simply to cut back on intramural budgets. Especially in turbulent times such as the years ahead are likely to be, there will be a continuing need for places and programmes at which researchers known to be productive can be kept at work. NIH's intramural programmes could marvellously complement those of external institutions if they were more able to employ people for short spells of time on projects whose purpose had been demonstrated to be productive.

The turmoil ahead for NASA is even bigger in scale (see page 266). Daniel Goldin, the head of the agency, seems to have borrowed freely from the proposed restructuring of the Department of Energy laboratories a few weeks ago (see *Nature* 373, 457–458 & 463–464; 1995). The idea is to hive off specific chunks of NASA's interest in science to "institutes" sponsored by universities, but initially supported by NASA. It seems unlikely, in the present climate, that NASA will be able to offer much in the way of long-term security to the people transferred or to the universities that take them on. It will be interesting to read the fine print in NASA's first agreement with a university. Only then will it be possible to be sure that the plan is not a way of dispensing with people whose cost can no longer be afforded.

Meanwhile, the universities themselves are not in particularly good shape. It is not a good sign that the University of California, for example, is persuading members of its faculties to take early retirement and then rehiring them to shoulder a reduced teaching load, largely because the academic pension fund is in surplus, but the state is broke. Much the same is true elsewhere in publicly funded higher education in the United States: the states are having to skimp on essential infrastructure just when the Republican revolutionaries are cutting back on indirect costs.

Unless the Congress is more careful than it has been in the past few months, there is a serious danger that it will seriously damage one of the best things the federal government has done in the past half century. But will not market forces ensure that the useful research is done (and that public money is not wasted on research that serves no purpose)? That is the Republicans' last defence. But precisely because the link between research and industrial utility is so intangible, that market is not an efficient market. Experience elsewhere, in Britain for example, shows that clearly enough. The Congress should put dogma to one side in its treatment of research. □

Xenophobia bubbles up

A Russian deputy's attack on Mr George Soros and his good works in Russia is an ominous and regressive sign.

THE Russian Duma (parliament) has not been a pleasing place for many years, but it seems to have outdone itself last week. The chairman of the Security Committee, Mr Victor Ilyukhin, whose role is to oversee the remodelled KGB, has written to *Sovetskaya Rossiya* with an account of his analysis of the work of Mr George Soros in Russia over the past few years. Ilyukhin is one of the old guard in the Duma, but his language is nevertheless an unpleasant echo of almost forgotten usage.

Thus, Ilyukhin says, many of the people employed by Soros in Russia to administer his two charitable funds are also "agents of the CIA", the US Central Intelligence Agency. He is particularly suspicious of the Soros scheme for making awards of cash to teachers and professors at Russia's schools and universities, which is intended to "change the mentality of Russian society". He fears "the degradation of social, patriotic and national consciousness" and worse: he goes on to accuse Soros (whose hedge fund made a killing from selling sterling short just before the British government's exit from the European Exchange Rate System in October 1992) of having manipulated the Russian ruble two years later, causing it to fall in value by a quarter. Ilyukhin now wants the Duma to legislate for strict regulation of foreign philanthropic organizations in Russia, especially for those with interests in the scientific field.

This is an unpleasant business because Ilyukhin means to be nasty. And his following in the Duma will listen to what he says, despite the counter-resolution being prepared by a member of Gaidar party in the Duma. It is also a convenient time to raise a protest of this kind. The Soros fund for science is now almost spent, so that it is unlikely that the fiercest resolution in the Duma will have much effect on the flow of funds to Russia. Yet Ilyukhin's protest is an ominous development. Russia's traditional habit is xenophobic. The survival of the tsars and their successors exploited that. It will be a shabby (and a self-depriving) business if it comes into bloom again. But true xenophobes do not believe that philanthropy can be disinterested and altruistic. They are their own worst enemies. □

UK goes for 'generic priorities' in bid to boost science/industry links

London. John Major, Britain's prime minister, has given his endorsement to a set of linked initiatives being launched by various government departments to stimulate technological innovation by building closer links between science and industry.

Speaking at a press conference in London on Monday, Major welcomed the report of the steering committee of the recent Technology Foresight exercise as "the next stage in our science and technology policy".

David Hunt, the minister responsible for science, announced that the government is to provide an extra £40 million (\$64 million) over the next four years — with the expectation that this will be matched by a similar sum from the private sector — to a new fund intended to support collaborative initiatives on priority topics identified by the Foresight programme.

In parallel, the Department of Trade and Industry (DTI), whose spending on research and development has — as the opposition Labour Party gleefully points out — fallen from £900 million in real terms in 1984–85 to £245 million in 1994–95, announced a slight reversal of this trend with an extra £70 million pledged to a variety of moves to promote technological innovation.

The high-profile press conference, attended by five cabinet ministers, was held to launch three separate documents which the government, whose moral and political credibility has recently been taking a severe battering, sees as the cornerstones of its science and technology policy.

The first was the report of the steering committee of the Foresight exercise, which was launched in the 1993 white paper on science, and resulted last month in the publication of the reports of 15 sectoral panels.

Out of 360 detailed recommendations from the panels, the steering group has identified six cross-sectoral strategic priorities. Three focus on the development of new technologies: in communications and computers; in developing new organisms, products and processes from genetics; and in advances in materials science, engineering and technology.

The remaining three priorities are more concerned with ways of exploiting new technologies. They emphasize the need to improve production processes through new technologies such as sensors and automation, to create a 'more sustainable world', and to address the impact of demographic change in creating new markets and ensuring the acceptability of technical change.

Within these strategic themes, the steering group has identified 27 'generic' priorities

in science and technology, subsequently grading these according to feasibility and attractiveness. Top of the list come genetic and biomolecular engineering, bioinformatics and 'communication with machines'.

The second document is the so-called *Forward Look*. This has been prepared by the Office of Science and Technology as an overview of the current research and development activities — and future plans — of all government departments. As such, it is intended to be a vehicle by which the government as a

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On target? Major (top) has welcomed efforts by Stewart (right) to focus UK research.

whole can judge the extent to which such plans coincide with its broad political and economic objectives.

Complementing these was a new DTI white paper on industrial competitiveness. Government officials claim that the general philosophy contained in the three documents forms a coherent strategy for ensuring the application of science to wealth creation and enhancing the quality of life without an excessive centralization of power.

In particular, they point out that preliminary findings from the Technology Foresight exercise have already been used by various departments in deciding their future spending plans on research and development.

For example, the distribution of additional funding made available to the six research councils in this year's science budget is already being distributed to topics — such as genome sequencing and 'cognitive engineering' — whose importance has been endorsed by one or more of the Foresight panels.

The trend is expected to increase. One proposal from the steering committee is that both universities and the Higher Education Funding Councils should reflect Foresight priorities in the way they allocate both recurrent and capital expenditure.

"At the end of the day, we have delivered", says Sir William Stewart, the government's chief scientific adviser and, as head of the Office of Science and Technology, one of the architects of both the white paper and Technology Foresight.

Reactions in industry have been mixed. Many, while welcoming the direction in which government policy is pointing, remain cautious until they are able to assess whether the new initiatives will make a significant impact on the thinking of investors and shareholders.

Caution is equally marked in the academic community. There is relief that Technology Foresight has not recommended a full-scale swing towards short-term objectives. The important of strategic priorities is also recognized — while the steering group's endorsement of universities' needs for modern equipment and the protection of long-term thinking is likely to be welcomed.

But there is also an awareness that, without promises of extra funding, greater support for priority areas will inevitably mean less money for those who have greater difficulty in persuading the government that their work contributes to wealth creation. "We must await careful analysis of the details to tell if the 'boosts' announced today are real new money, or repackaged old money," said a statement from the pressure group Save British Science.

Perhaps inevitably, the greatest scepticism has come from the Labour Party. John Battle, the party's science spokesman, points out that the new money being allocated to collaborative research and development efforts is relatively small compared to the cuts in government R&D spending over the previous few years.

Battle also says that, in practice, the degree of harmony between the research plans of government departments is considerably less than the OST claims, and pledges that, if elected, Labour will remedy the situation with its own plans for greater co-ordination.

But the government remains convinced that it is on the right track. "The Technology Foresight exercise in the United Kingdom is seen as a trailblazer across the world," said Hunt on Monday. A further report on the programme's progress will be published at the end of the year.

David Dickson

Job losses loom in NASA restructuring plan

Washington. Scientists employed by the US National Aeronautics and Space Administration (NASA) would find themselves working for government-funded science 'institutes' under a restructuring plan unveiled by Daniel Goldin, NASA's administrator, last week.

The institutes, whose shape has been under review for several months (see *Nature* **374**, 107; 1995), would be run by universities, consortia or private companies. Goldin says he does not expect the move to save money. But he says it will lead to "much stronger science for NASA" by leading to an increased use of peer review and greater cooperation with academic and industry scientists.

The proposed science institutes are only part of a wide-ranging restructuring of the agency that has been drawn up over the past year. Among other changes, the space shuttle would be turned over to a single contractor, with an eye to eventual privatization.

Overall, says Goldin, between 25,000 and 30,000 government and contractor employees will lose their jobs, although no centres will be closed and no major projects terminated. The scaling-back is the result of reduced NASA budget projections published by the White House in January.

The science institutes would be carved out of existing programmes at the agency's 11 centres, and would use existing NASA facilities; Goldin has ruled out any new "brick and mortar". The Ames Research Center near San Francisco, for example, would house a new institute for astrobiology, and the Johnson Space Center near Houston will have institutes for biomedical and planetary science.

An atmospheric science institute will be set up at the Langley Research Center in Virginia, one devoted to microgravity and space power at the Lewis Research Center

in Cleveland, and one for hydrology and other science at the Marshall Space Flight Center in Alabama.

The Goddard Space Flight Center in the Maryland suburbs of Washington DC will remain the agency's main centre for Earth science, physics and astronomy. The Jet Propulsion Laboratory in Pasadena, California will retain primary responsibility for Solar System exploration.

According to Goldin, planning is already under way for the first institute — the space biomedical research centre at Johnson — and the head of a team being set up to oversee the transition from NASA's current biomedical science operations should be named by next week. Potential operators for the new institute include the Texas Medical Center.

Goldin said the agency will go slowly in setting up the institutes, using Johnson as a pilot project. He and France Cordova, NASA's chief scientist, have been holding meetings with university deans, state governors and other officials, looking for potential institute sponsors. Cordova says she already has "a portfolio a couple of inches thick" of letters expressing interest in cooperating with NASA.

Even though many details still have to be worked out, Cordova hopes one or two institutes will be in place by 1997. Last week she met science directors from the NASA centres to begin working out the details of how the institutes will be opened to competition for sponsors, how the contractual status of

NASA scientists will be organized, and other administrative matters.

Cordova says the institutes will involve a "more diverse, larger community" than NASA science laboratories do at present. "We're not trying to shape the institutes in NASA's image," she says.

In general, the idea of setting up privatized science institutes has been welcomed by the space science community. Anneila Sargent of the California Institute of Technology, who chairs NASA's science advisory committee, admits that some NASA science positions will be lost in the shuffle. But she says that considering the inevitability of cuts at the agency, "space science came out [of the restructuring] rather well".

Nevertheless, NASA's problems may be far from over. No sooner had Goldin announced the plan last week than he was fending off yet another attack on his budget from Congress. Further deep cuts proposed by the House of Representatives (see *Nature*, **375**, 168; 1995) would have a "devastating effect on NASA centres and programmes", he warned, and would almost certainly lead to the closure of centres and cancellation of projects.

One likely victim is the agency's Earth Observing System (EOS) satellite programme, which many Republicans see as little more than Vice President Al Gore's pet environmental project, and which has been drastically cut in the House budget plan.

An internal NASA memorandum being circulated in Washington last week speculated that, if the space agency had to meet the new House targets, "the common [EOS] spacecraft procurement could be slid to accommodate the funding profile," and the programme's sophisticated data distribution system "could be cancelled or completely restructured".

Tony Reichhardt

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Goldin: promises 'much stronger science'.

NIH will reallocate research funding to support DNA sequencing

Washington. Harold Varmus, the director of the US National Institutes of Health (NIH), said last week that he proposes to use the discretion he has been given by Congress in allocating a small amount of NIH funds to support new research initiatives in three areas; developing DNA sequencing technology, preparing a map of the rat genome and techniques for small-angle X-ray scattering and spectroscopy.

Following the first meeting of a panel of experts he has set up to advise him on priorities in gene therapy research, Varmus said that, in the light of impending budget cuts under discussion in Congress, "we can't infuse huge sums of money into any new programme". But, he added, "I can move money around".

At the same meeting, Francis Collins,

director of NIH's National Center for Human Genome Research, made a plea for the development of mechanisms for funding imaginative research on new potential vectors for gene therapy. "Perhaps we need to try some completely new virus, rather than taking small, incremental steps," he said. "There are too many reasons not to always take a safe approach."

The conclusions of the panel, set up explicitly to assess NIH's investment in research in gene therapy, are expected to influence how Varmus allocates a discretionary fund amounting to 1 per cent of the total NIH budget that was authorized by Congress in its appropriations for the fiscal year 1995, which begins on 1 October. At present, NIH spends \$135 million a year on extramural research in gene therapy, and

\$61 million on its intramural programme.

The advisory panel, co-chaired by Stuart Orkin of Harvard Medical School in Boston and Arno Motulsky of the University of Washington in Seattle, heard about many promises, but few effective long-term therapies, in the quest to move gene therapy out of the laboratory and into the clinic. Several participants at last week's meeting said that current difficulties suggest more basic research may be needed before large-scale clinical trials are launched.

Their comments were prompted by the fact that, although many gene therapy protocols have yielded promising results in the early stages, these are often overshadowed by lacklustre or inconsistent results in clinical trials.

According to Collins, one major hurdle to

Review proposes shift in US cancer funding

Washington. Sharp cuts in the share of research funds allocated to the intramural programme at the US National Cancer Institute (NCI) have been recommended by a panel of prominent cancer researchers. The recommendations are likely to become a blueprint for retrenchment.

The 12-member panel of university researchers has also proposed reshuffling the contents of NCI's four divisions — each of which is responsible for both intramural and extramural research on different facets of cancer biology and treatment — into two divisions, one for intramural and the other for extramural research.

Both conclusions have emerged from seven months of deliberations, and were presented last week by the panel's two chairmen, Paul Calabresi of Brown University in Providence, Rhode Island, and J. Michael Bishop of the University of California, San Francisco, to a meeting of the National Cancer Advisory Board (NCAB) on 16 May. (The full text of the panel's report will be released in mid-June.)

Other recommendations include: strengthening peer review and strategic planning of NCI's intramural research, and spinning off a significant portion of its AIDS research to sharpen the focus on cancer.

At present, NCI channels 18 per cent of its \$2-billion annual budget into its intramural programme. But, according to Bishop, that proportion increases to a massive 25 per cent when money spent on research contracts that directly support the intramural programme is included.

The latter level of support contrasts starkly with the average intramural spending of 11.3 per cent across the whole of the NIH. The panel agreed that "no single site should have such a large fraction of the federal investment in cancer research," says Bishop.

work, says director

developing safe and reliable gene therapies is the lack of suitable vectors for introducing genes into cells. "Think about how the modern day bicycle evolved from the Victorian era model," said Collins. "The basic structure was flawed — it needed a major overhaul. With gene therapy we may be arguing about the number of spokes that should be in each wheel, rather than whether we have the ideal basic design in place."

Collins said he hoped the panel "will encourage people to take chances" in working on novel vectors. Varmus, who has previously expressed concern that gene therapy may be promising more that it can deliver in the near future, told the panel that "maybe we need to let basic research percolate a bit before moving too quickly into large-scale clinical trials".

Nancy Touchette

The scrutiny comes at a difficult time in NCI's history. Harold Varmus, director of NIH, and the then NCI director Samuel Broder convened the panel late last summer in response to the call from another advisory committee for a review of the intramural programmes at each NIH institute.

Meanwhile, several senior NCI officials, including Broder (who has expressed his own unhappiness at proposals to reduce the intramural programmes), have resigned and their positions remain unfilled — although Broder's position is expected to be filled in the near future by Richard Klausner, a cell biologist at NIH.

Moreover, changes in healthcare financing are making the conduct of clinical research more difficult, as health maintenance organizations are increasingly reluctant to refer patients to the relatively more expensive hospitals where research is being carried out. And budget pressures in Congress are adding to the problem.

In this context, most researchers are likely to support the panel's conclusions, if only because they have become resigned to shrinking budgets. "If you start with the agenda that NCI must be pruned, these kinds of recommendations are appropriate," says Robert Gallo, chief of the NCI Labora-

tory of Tumor Cell Biology.

Bruce Chabner, who left his position as director of NCI's Division of Cancer Treatment in April and is now at Harvard University and Massachusetts General Hospital, says that although he does not agree with everything in the plan, "it is a reasonable attempt to reorganize the institute".

But Chabner adds that the plan's ultimate success "will depend on the people hired to execute it and the budget they are given". One concern among NCI staff is that a greater centralization of its work into two divisions could make both more vulnerable to budget cuts. Another is that any significant reduction in intramural research spending could inevitably lead to job losses among researchers at the institute.

Expressing its own form of caution, the NCAB unanimously voted to accept the panel's report "in spirit" — but reserved judgement on the details of each specific proposal. Barbara Rimer, of Duke University Medical School in Durham, North Carolina, who chairs the advisory board, said that the panel members had produced a "brilliant and comprehensive report", but that it is now up to the institute — and in particular its new director — to implement it.

Robert Taylor

NIH backers pledge last-ditch stand

Washington. A small but influential group of moderate Republican senators have launched a bid to save the National Institutes of Health (NIH) from proposed budget cuts which, according to NIH director Harold Varmus, could slash the success rate for grant applicants next year from 24 per cent to "between 6 and 12 per cent".

"We may fail, but at least we'll die with our boots on," said Mark Hatfield (Republican, Oregon), chair of the Senate appropriations committee, last week. Hatfield was pledging his opposition to cuts of at least 10 per cent in NIH's \$11.3 billion budget which were proposed two weeks ago by the rival Senate budget committee, chaired by Pete Domenici.

On Monday, Hatfield was trying to draft an amendment to the Senate budget resolution which would win the Senate's approval for a bid to fully restore proposed NIH cuts of \$7.7 billion over the next five years. The amendment would specify alternative targets.

Most of the 46 Democrat senators were likely to support the amendment. But Hatfield needed the support of at least 5 Republicans if the amendment was to pass. He was expected to get the support of two leading Senators who chair committees with oversight of NIH — Arlen

Specter (Republican, Pennsylvania) and Nancy Kassebaum (Republican, Kansas) — but other Republicans were proving difficult to recruit.

Biomedical lobbyists, aware of the possibility of defeat, have been playing down the significance of the vote, noting further opportunities to restore NIH funding cuts will arise later in the budget process.

At a hearing last week of the Labor, Health and Human Services and Education appropriations subcommittee, chaired by Specter, Varmus and his team of institute directors were asked to produce brief 'sound-bite' length assessments of the impact that such a cut would have.

In response, Varmus said the cut would cause the success rate of grant applicants to collapse. But Specter appeared impatient with the NIH team and what he interpreted as their failure to express their plight in sufficiently bald terms.

Although most members of the panel were sympathetic to NIH, Herb Kohl (Democrat, Wisconsin) called on Varmus to "eliminate inefficiencies" at NIH. For example, Kohl wanted to know why it had 700 people working for the director's office, and why the budget of the National Library of Medicine has expanded by \$35 million.

Colin Macilwain

Agency heads say sorry to board for criticism from staff

Sydney. In a new twist to the troubled relations between scientific staff and top managers at Australia's largest research agency, the Commonwealth Scientific and Industrial Research Organisation (CSIRO), the heads of the agency's five major research divisions have apparently written to apologize for criticisms by their staffs of the CSIRO board.

The letters, which were subsequently read out to the staff of the five divisions, were written after a meeting between Roy Green, acting chief executive of CSIRO, and the heads of units based around the national capital, Canberra.

None of the unit heads — nor Green — would comment last week on what happened at the meeting, which had been held to discuss a submission to the board signed by most of the Canberra staff after a management report suggesting various structural changes had been circulated for comment (see *Nature* 374, 587; 1995).

The staff document complained of a "widespread lack of confidence" in both the board and a committee it had set up to produce the management report. But after discussing its strong wording with Green and another board member, the division heads all agreed to send letters that those who heard them read out say amounted to apologies.

According to one CSIRO scientist, the main source of contention was not the remark about a lack of confidence but a separate accusation that board members had a "conflict of interest". The staff had intended this merely as a comment on the fact that board members are part-time — only the acting chief executive officer is full-time — and hold posts outside the organization, not that there is any monetary conflict.

Green later denied that the letter was an apology, but said that the wording in the submission did not appear to convey the message that the staff had wished to communicate to the board. He said that the division chiefs had acknowledged that point — and had so informed their staff.

Both Green and Adrienne Clarke, chair of the CSIRO board and professor of botany at Melbourne University, say they accept that the present staff difficulties stem from failures of internal communication. Another widely cited problem is the amount of time taken up with administrative requirements and frequent reviews of research.

Green is now setting up a committee to examine such issues. At the same time, he denies having ignored the report of a previous Senate committee of inquiry on CSIRO, and says that it was taken into account during the writing of CSIRO's own management report.

Mark Lawson

Coalition plans challenge to genetic patenting in the US

San Francisco. Scientists, clergy and activists will meet in the Adirondack mountains next week to discuss how to stimulate a broad public debate in the United States about the moral and ethical aspects of patenting human genes and living organisms.

The meeting, which will also be attended by representatives of agricultural, native American and health advocacy groups, comes on the heels of a well-publicized appeal by a broad-based coalition of more than 175 religious leaders to stop the patenting of genetically engineered animals and human genes, cells and organs.

The statement received front-page coverage in newspapers across the United States. It brings together a number of weighty political constituencies that often find themselves on opposite sides of issues such as abortion and school prayers.

The campaign has been largely organized by Jeremy Rifkin, director of the Foundation on Economic Trends in Washington, and a long-time opponent of genetic engineering. The signatories say that they are not concerned about the biotechnology industry itself. But they voiced strong disagreement with attempts by scientists and corporations to "grab" what they consider rightfully belongs to humanity.

"We have to consider [the human genome] to be a natural resource," said Abdurahman Alamoudi, executive director of the American Muslim Council in Washington. "It's like the water, the wind; it doesn't belong to anybody."

Biotechnology industry leaders have responded by arguing that patents are essential for the development of modern medicines, and by drawing a distinction between the legal protection conferred by patenting and actual ownership.

But members of the Council for Responsible Genetics, which is organizing next week's meeting, say that patents on living organisms may impede medical progress by effectively 'privatizing' publicly funded research and limiting the free exchange of scientific information.

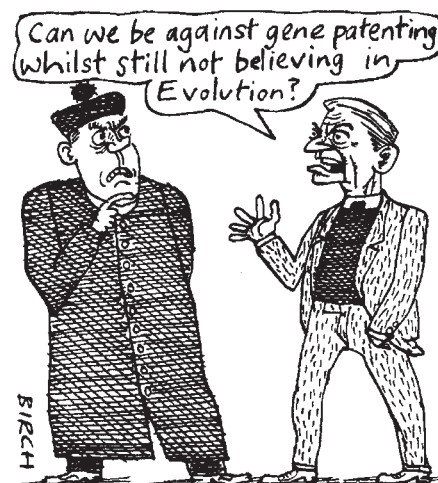
Richard Levins, John Rock professor of population science at the Harvard School of Public Health, called gene patenting a means of "subordinating a common intellectual heritage for private gain". He and other scientists say that the traditional collegial exchange of ideas in laboratories is being replaced by a concern for protecting potential monetary gains.

Philip L. Bereano, professor of public policy at the University of Washington and an organizer of next week's meeting, says that the decision by religious leaders to take

up the issue is likely to help awaken the American public to the debate.

The religious leaders themselves said last week that they hope the US Patent and Trademark Office will suspend the issuing of new patents. They plan to begin discussions with their own members, and to put pressure on Congress to take action.

Americans have ceded decisions on issues raised by biotechnology to commercial and scientific interests, said Rabbi Daniel Swartz, director of congregational relations at the Religious Action Center in Washington. He said religious leaders can put the



gene patents in the context of the broader devaluation of life in modern society.

But many scientists feel that the clergy's petition reflects either a lack of understanding of the patent system or a bias against biotechnology in general. David Galas, for example, vice president of research and development for Darwin Molecular Corporation in Seattle, says the call for a moratorium is far too sweeping and could crush the financial incentive behind the industry.

Galas, who opposes patenting of gene sequences whose function is unknown, points out that patents are designed as a way of simultaneously placing information in the public domain, and rewarding the inventor.

Rebecca Eisenberg, professor of law at the University of Michigan law school and an adviser to the National Institutes of Health on patent issues, acknowledges that biotechnology patents raise important issues. But she says that Rifkin's group poses the question in a sensationalist and provocative manner.

Instead, says Eisenberg, the public should be looking at the impact of the current proliferation of patents on basic research, and whether patenting the early stages of a technical development retards later commercial development.

Sally Lehrman

Chinese academics share human rights prize

London. Two eminent Chinese intellectuals, who last week were involved in drafting and circulating a petition to the government calling for an amnesty for those involved in the 1989 pro-democracy demonstrations, are to share a human rights award conferred by the New York Academy of Sciences.

In an announcement due to be made today (25 May), Xu Liangying, a 75-year-old retired professor who has edited and translated the collected works of Einstein, is expected to receive this year's Heinz Pagels Award for the advancement of the human rights of scientists.

He will share the prize with Ding Zilin, a professor of philosophy at the People's University in Beijing. Her 17-year-old son died on the first night of the government crackdown on pro-democracy demonstrators in Tiananmen Square in 1989.

Past recipients of the award, to be presented later this year, include the late Andrei Sakharov, the former Soviet dissident physicist, and Fang Lizhi, a prominent Chinese pro-democracy activist who is now professor of physics at the University of Arizona.

Xu Chenggang, Xu Liangying's son who is an economics lecturer at the London School of Economics, says the award "is a great honour for my father". He adds that "it will mean a lot, especially at this time."

Meanwhile, government authorities in China reacted with predictable hostility to the petition drafted on 15 May by Liangying and signed by 45 intellectuals, including Wang Ganchang, the 88-year-old physicist who played a leading role in China's fission programme.

The signatories also include 15 members of the Chinese Academy of Sciences and eight women professors. The letter was delivered to both President Jiang Zemin and Qiao Shi, chairman of the National People's Congress, China's parliament.

Five signatories have already been arrested, including Wang Dan, a prominent student leader, and Huang Xiang, a leading poet. The rest are said to be under police surveillance.

Communist Party officials have visited the houses of at least two of the scientists, urging them to withdraw their names from the letter. Also under arrest is Liu Xiabo, a literary critic who is responsible for circulating a separate letter to the government.

The American Physical Society, which recently established official contact with its Chinese counterpart, has written to the Chinese President, Jiang Zemin, expressing concern over reports that Wang Ganchang is one of the scientists being harassed.

According to authoritative sources in Beijing, Communist Party officials are said to have questioned the octogenarian for three hours. They then refused to leave his residence, or allow him to sleep until he signed

a statement saying his signature on the petition was a mistake.

The sources said Ganchang "steadfastly refused to sign". But unconfirmed reports suggest he may have been persuaded to put his name to another statement saying he was not aware that the petition would be published overseas.

Xu Liangying said in a telephone interview from his home in Beijing that his mail is being intercepted, although there are as yet "no restrictions" on his movements. "I can come and go wherever and whenever I



Xu Liangying (left) meets with former award-recipient, physicist Fang Lizhi (right) and his wife Li Shuxian (centre).

want," he said through an interpreter, adding that three police cars stationed outside his house "have not stopped me from anything".

Nevertheless, the telephone conversation was cut off at least half-a-dozen times, and Liangying emphasized that contact with foreign journalists was being made progressively more difficult.

The letter's signatories are the highest-ranking group to have defied a government ban on organized protest since 1989. They use as the letter's theme a United Nations resolution proclaiming 1995 as the UN's Year of Tolerance.

The petition, specifically, urges the authorities to "treat all views such as ideology, political thought and religious belief with a spirit of tolerance and never again regard individuals of independent thought

and views as 'hostile elements' and submit them to repressive attacks, surveillance, house arrest, even detention". It also calls for a re-evaluation of "the 4 June 1989 incident, seeking truth from fact and release those people who remain in jail".

Despite the letter's link with the UN Year of Tolerance, observers believe its timing may have been influenced by a number of additional factors. Two recent government announcements are likely to have been in the signatories' minds, says Xiao Qiang, a spokesman for Human Rights for China, based in New York.

One was a decision to mount an official crackdown on corruption in China's public sector after the mayor of Beijing, Chen Xitong, resigned four weeks ago, following the suicides of two of his deputies who had been implicated in a corruption scandal. The second was a government statement promising to treble the country's science budget by the year 2000 (see *Nature* 375, 175; 1995).

"The scientists who signed the petition say it is meaningless to have campaigns against corruption or promises to increase the science budget, when there is no human rights or democracy," says Qiang, an astrophysicist by training who now works as a human rights activist.

"When the government makes such pronouncements, it usually means the situation is getting dire," he says. "Scientists welcome the money, but they find it difficult to be motivated without guarantees for their rights," adds Xu Chenggang.

Another observer, however, believes that the petition's timing may also be related to the disarray over the succession to the ailing elder statesman Deng Xiaoping. "My guess is that these scientists wanted to test the water," says Steve Tsang, a fellow at the Centre for Modern Chinese Studies at St Antony's College, Oxford.

"A not-too-brutal response might be an indication that the government is too divided; meaning this is a good time to push for a reversal of the verdicts handed down on those who took part in the 1989 demonstrations."

Ehsan Masood

Amazon scientists seek funds for Internet link

London. Scientists working in the central Brazilian Amazon have issued a plea for funds to save their solitary Internet connection, which is due to be severed by Embratel, the Brazilian telephone monolith on 12 June.

The satellite link is to be cut as the Rede Nacional de Pesquisa — the Brazilian Internet — says it is no longer able to continue paying the \$15,000 monthly connection bill.

The link is used extensively by scientists working for the Instituto Nacional de

Pesquisas da Amazonia (the National Institute for Amazonian Research). But the state of Amazonas contains less than two per cent of the country's population, making the Internet link the country's most expensive.

A source at the Brazilian Internet said another reason for cutting the link could be the impending commercialization of Internet access in Brazil. "The Rede Nacional de Pesquisa is at this moment in the middle of heated negotiations with Embratel," he said.

Ruling brings cash windfall in US fraud case

San Diego. In a novel lawsuit, a New York scientist last week won a \$1.9 million-judgment against a group of Alabama researchers who were found by a jury to have stolen study results and used them to improperly obtain federal grants.

The lawsuit, which was heard in federal court in Baltimore, Maryland, is thought to be the first case in the United States where a jury has ruled that academic scientists stole research results from another scientist.

In last week's case, Pamela A. Berge, an epidemiologist, successfully sued three paediatricians and a junior researcher at the University of Alabama at Birmingham for stealing her work and presenting it to the National Institutes of Health (NIH) as their own. The paediatricians, all physicians, are Robert Pass, Sergio Stagno and Charles Alford. The junior researcher, Karen B. Fowler, is now an assistant professor.

On 1 May, a federal jury in Baltimore found in Berge's favour. And on 16 May, US District Judge Edward S. Northrop ordered the University of Alabama to pay \$1.66 million for making false claims to the NIH. The US government will receive \$1,162,000 and Berge will receive the remaining \$498,000, as well as an additional \$265,000 from the Alabama scientists for misappropriation of her research, of which \$215,000 is punitive damages. The University of Alabama has also been ordered to pay a \$10,000 fine in addition to Berge's legal costs.

The Alabama physicians are renowned for their 17 years of work on cytomegalovirus (CMV), a genital viral infection that can cause eye, ear or neurological impairment in newborn infants. They were laying the groundwork for a vaccine to prevent mother-to-child infection with the virus. The NIH continues to provide about \$1 million annually to the Alabama research team. NIH officials say they are assessing the legal case to determine what, if any, action they should take.

Neither the Alabama researchers — who denied any impropriety in documents submitted to the court — nor their attorneys would discuss the verdict. But the university's general counsel, Ina Leonard, issued a brief statement on 18 May saying the university "is extremely disappointed about the jury verdict". Leonard said those convicted intend to appeal. "We believe the verdict is wrong and that it is not consistent with or supported by the evidence presented."

For Berge, now 47 and a consultant at Pfizer Inc., the entire affair remains baffling. "It has been a nightmare," she said. Berge, who has since lost her academic job, says she now feels smeared for having challenged her collaborators. "I am appalled at what happened. I don't understand the motive. I don't understand why it had to happen."

Berge was a doctoral student at Cornell

University in New York in 1987, and spent nearly a year in Alabama, collaborating with the Birmingham group. She had alleged that the Alabama team gave data from her research to Fowler, then a doctoral student. She accused Fowler of plagiarizing her work, misusing her methodology and then using the research for her 1990 dissertation and two articles published in 1991 and 1993.

Berge also alleged that the Alabama team repeatedly over several years made false representations to win continuing grants from the NIH. The Alabama team, she claimed, misrepresented the status of the project to the NIH, omitted results that contradicted its theories, and claimed Berge's work as their own.

The case was tried under the so-called 'whistle-blower law', a federal statute that allows those who expose fraud in government to receive a share of any money recovered in subsequent litigation.

The law was enacted primarily to combat fraud in the defence industry, but it has recently been used in a number of cases to expose the misuse of grants from the

National Institutes of Health (NIH).

According to several government and legal authorities, Berge's lawsuit is the first case under the whistle-blower law involving allegations of scientific misconduct that was decided by jury. (The other cases were dismissed or settled by out-of-court payments.)

US researchers are increasingly turning to the courts to resolve disputes that have historically been addressed in academic institutions — a trend that is already producing considerable debate over methods for handling scientific misconduct cases.

Those who advocate use of the courts to decide such scientific disputes say universities and government funding agencies have failed to handle complaints of wrongdoing fairly and effectively. One result is that academic reviews can drag on for years, damaging the careers of both the accuser and the accused.

But others say the courts are the wrong place to adjudicate such disputes, as the outcome is more likely to depend on the skill of attorneys and knowledge of legal procedures than on factual evidence. **Rex Dalton**

Costs discourage European patents

Munich. High administrative fees and translation costs are discouraging small and medium-sized firms in Europe from filing patents, according to the results of a survey published last week by the European Patent Office in Munich (EPO).

The EPO commissioned the survey following continued complaints from industry that the costs of patenting through the EPO were higher than either through national patent offices in Europe, or through the United States Patent and Trademark Office (USPTO) and the Japanese Patent Office (JPO).

In recent years, European firms have been filing increasingly fewer patent applications — although the number last year was slightly higher than in 1993 — particularly in key technologies, where most patents are now owned by non-European companies (see chart).

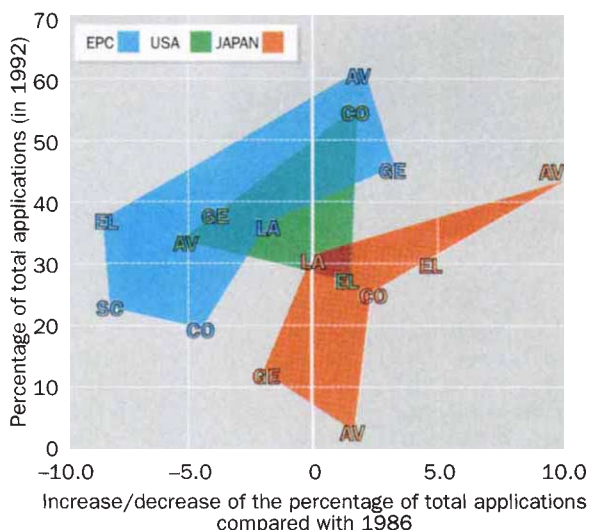
The EPO is keen to identify ways of changing this situation by reducing the costs of patenting. But the true costs have been difficult to estimate, as they are shared between the applicants, patent offices and patent attorneys. It is also difficult to make international comparisons, as the level of patent

protection is not identical in each country.

Companies with their own patent departments, national patent offices and patent attorneys were asked by the EPO to estimate their expenses during 1992. The results show that the true total cost of an average EPO patent, including translation costs and official fees, amounts to DM45,510 (US\$32,500) for registration in the eight most designated member states.

This price does not include the annual

Changing sources of European patent applications



CO-Computing **SC**-Semiconductors **LA**-Laser **AV**-Aviation
GE-Genetic engineering **EL**-Electronics/communications

renewal fees which the applicant must pay to the national patent offices in the designated countries to keep the patent alive (half of these fees are returned to the EPO).

The survey concludes that it is still cheaper, as intended in the European Patent Convention of 1973, for a company to patent an invention through the EPO, designating protection in the three largest member countries — namely the United Kingdom, Germany and France — than filing individually in the three countries. This is primarily because only one set of attorney fees is involved, instead of three. But the margin is only six per cent.

The survey found that the average cost of filing a patent in the United States or Japan is around 20 per cent cheaper than the EPO. This is because, although the attorney fees are much lower in Europe, the official patent office fees, at DM9,900, are at least three times as high.

Another major item of expenditure for European patents that is not shared by Japan and the United States is translation costs. Patent applications can be submitted in one of the three EPO official languages, namely English, French or German, with the summarized claims being translated into the other two languages on publication.

But EPO member states insist that any EPO patent given protection in their country must be translated in full into the national language. This means that for an average patent, covering eight designated member states, translation costs constitute on average DM15,200, or one-third of the total cost.

The EPO is keen to reduce the costs associated with translation. But doing so requires the political agreement of its member states. Although translations have no legal status, attempts in the past to eliminate their requirement have been resisted by member states, who insist that it is the only way to guarantee their citizens full access to patent information.

The EPO administrative council has highlighted suggestions for cost saving in a discussion document made public last week. The document invites comments from both researchers and industrial representatives on other cost-saving proposals, such as reducing the duplication of work between the EPO and national patent offices, for example by centralizing of some activities such as translation.

Another suggestion would be to devolve responsibility for activities such as searches to the larger national offices. But this is a politically-sensitive issue because of the continuing power struggle between the national and European offices.

Furthermore, any conclusions based on the comments received could take a considerable time to implement. As EPO staff members despairingly note, the conflicting interests of member states have frequently in the past prevented apparently obvious solutions to the EPO's problems from being introduced.

Alison Abbott

Chirac downgrades science in re-shuffle of ministries

Paris. The incoming government of France's new president, Jacques Chirac, has stunned the country's researchers by abolishing France's independent science ministry. The change ends the prominent political status given to research under France's Socialist president François Mitterrand during his 14-year period of office.

"It's a step backwards of about 20 years for research," said one leading French research official in reaction to news that the administration of Alain Juppé, the new Prime Minister, will fold research into a superministry of "national education, higher education, research and social insertion".

Under the governments of Mitterrand, research either had its own ministry, or was combined with higher education or with industry. Observers fear that the new arrangements could result in research being neglected because of the enormous day-to-day demands of the school and higher education portfolios.

The nomination of a secretary of state for research has done little to allay such concerns. Unlike ministers and junior ministers, secretaries of state do not routinely attend cabinet meetings, for example.

Moreover, while research was run by a secretary of state under Giscard d'Estaing's presidency during the 1970s, the post was directly attached to the prime minister. It is now attached to the education ministry.

The lack of a cabinet minister dedicated to research could, in particular, damage France's ability to develop both a national strategy for research, and its international policy, particularly in Brussels, argues Pierre Papon, chairman of the Observatoire de Science et Technologie, and a former head of the Centre National de la Recherche Scientifique (CNRS).

Moreover, the new secretary of state, Elisabeth Dufourcq, 54, is relatively unknown in science policy circles. She is a researcher at INSERM, the national biomedical research agency, a member of the national bioethics advisory committee, and a historian who has published several books on women missionaries.

Observers argue that while the appointment of a secretary of state for research may improve day-to-day management of the research portfolio, Dufourcq lacks the political clout of past research

ministers such as François Fillon or Jean-Pierre Chevènement — who were both career politicians.

Nor will she start her new role with the reputation of the geophysicist Hubert Curien, who, although not a politician by profession, had headed both the French space agency (CNES) and CNRS, and was considered to be more wily than many professional politicians.

Some fear that the lack of such political punch will mean bad news for research, particularly when budget levels need to be negotiated with the powerful finance ministry. "My overall impression [of the new ministerial configuration] is that the political weight of research has been very strongly diminished," says one observer.

Edouard Brezin, the president of CNRS, argues that it is too soon to judge the new government's commitment to research. Although admitting that he would have been "more reassured" by an independent research ministry, he says he "hopes research will be one of the government's priorities."

In contrast, the SNCS, one of the labour unions that represent researchers, criticized the "drowning" of research within the new "superministry", as "a bad omen for the attitude of the new government to research".

While some argue that research is unlikely to face the harsh cuts it experienced when Chirac was prime minister in 1986, few believe he will keep his campaign promise to increase research spending.

They point out that during his election campaign, Chirac stated in an interview with *La Recherche* magazine that he favoured a dedicated research ministry, arguing that while the links between research and the universities were important, research had many aspects, and went far beyond the university.

The new minister who will hold ultimate political responsibility for research is François Bayrou, who was also minister of national education in the previous government. Bayrou visited CNRS headquarters last weekend immediately after his nomination, along with Dufourcq, in what is being seen as an attempt to allay concerns over the future of the cash-strapped agency (see *Nature* 374, 666, 1995). "Research will be at the centre of my preoccupations", he said, adding that he intended to make regular visits to CNRS.

Bayrou emphasized the "tight links" between research and education. He added that CNRS "assumes one of the grand functions of the nation, and is at the heart of one of the grand ministries of the Republic: this is a message of respect, and, I hope, of hope."

Declan Butler

IMAGE
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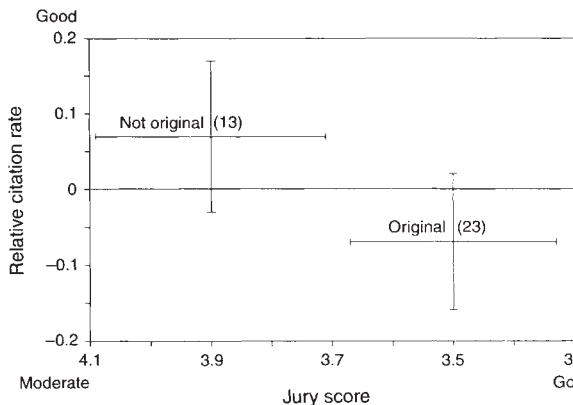
Bayrou: keen to allay concerns over CNRS.

Gamma

Evaluating research proposals

SIR — Peer review is widely used for evaluating research proposals. But bibliometric indicators are also occasionally used. We assume that highly qualified teams will submit high quality proposals, which will be recognized as such by the peer review procedure of the Dutch Technology Foundation (STW). We expect these highly qualified teams to have high visibility in international journals, their publications having a relatively high rate of citation. And we expect that these highly qualified teams will come up with original research proposals.

Working with a random sample of 75



proposals (relating to application-orientated research) submitted to STW between 1989 and 1990, we compared the results of peer review (as practised by STW) with the results of citation counting analysis (as practised by the Centre for Science & Technology, CWTS). We were curious to discover whether the two methods would give approximately the same ranking for originality.

CWTS collected bibliometric data about the publications of the team members who had submitted the 75 proposals and calculated citation-based indicators. CWTS worked with two particular indicators: citations per publication (CPP) and the journal citation score mean (JCSm). CWTS calculated, first, the average CPP for the members of each research team over a three-year period, and, second, the JCSm over all the journals in which team members had published. A 'relative' citation rate was arrived at by log (CPP/JCSm). This rate shows whether the team members' performance is lower (score <0) or higher (score >0) than the international average for the field. For 14 teams out of the 75, CWTS found no citations, so the relative citation rate did not exist. These proposals were not included in the further analysis.

STW's system, introduced in 1981, operates as follows: when a research proposal is submitted, at least five peers are invited to give well-founded comments. The principal investigator is asked to react

to these comments. Next, a jury of 12 people, all highly qualified but not experts in the field of the proposal, is required to rate a group of 20 proposals on the basis of the peer comments and the principal investigator's defence. The jury ratings are then averaged to obtain a score for the scientific quality and the potential usefulness of each proposal. Only the scientific quality marks are referred to as 'the jury score'. The jury score can range from 9 (poor) to 1 (excellent).

On the basis of the detailed peer comments, STW divided the proposals into three categories: 'original', 'not original' and 'in between'. For proposals to be ranked as 'original' and 'not original', the five peers had to be unanimous in their verdict (which is given independently of each other).

The figure summarizes the results of the two methods. As can be seen, there is a considerable discrepancy: the jury score for the research proposals was 'good', whereas the 'relative' citation rate was only 'moderate'.

The conclusion of this study must therefore be that although citation rates may well be valuable indicators of the impact of a team's past performance, they are not necessarily reliable indicators of the originality of research proposals, particularly when these relate to application-orientated research.

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Whaling error

SIR — You published recently (*Nature* 374, 587; 1995) a report headed "Error re-opens 'scientific' whaling debate".

The error in question, however, relates to commercial whaling, not to scientific whaling. Although Norway cites science as a basis for the way in which it sets its own quota, scientific whaling means something quite different, namely killing whales for research purposes. Any member of the International Whaling Commission (IWC) has the right to conduct a research catch under the International Convention for the Regulation of Whaling, 1946. The IWC has reviewed new research or scientific whaling programmes for Japan and Norway since the IWC

moratorium on commercial whaling began in 1986. In every case, the IWC advised Japan and Norway to reconsider the lethal aspects of their research programmes. Last year, however, Norway started a commercial hunt in combination with its scientific catch, despite the IWC moratorium.

Norway is not bound by the IWC moratorium because it entered an objection when the moratorium was adopted. Early this year, the government of Norway announced that it was setting a commercial quota of 301 minke whales for the 1995 season. (No research catch is scheduled for 1995.) The 1995 hunt of 301 whales is the same number as was set in 1994 for the combined research and commercial catches. Norway's commercial whaling quota is said to be calculated using the IWC's Revised Management Procedure (RMP) combined with the Scientific Committee's 1992 abundance estimate of 86,700 minke whales in the northeast Atlantic. The RMP, on which the IWC Scientific Committee (SC) has worked for a number of years, was accepted in principle by the IWC Commission in 1994, but it has not yet been applied to quotas for commercial whaling. Norway's setting of a commercial quota is completely independent.

The abundance estimate of northeast Atlantic minke whales is now in question (as noted in *Nature* 374, 587; 1995). At this year's meeting of the IWC SC, the methodology for estimating the abundance of this population will be reviewed in detail. Now that this abundance estimate is in question, Norway (using a new abundance estimate of 69,900) has reduced its 1995 quota by 23 per cent to 232. In fact, Norway's commercial hunt started on 2 May (six days before the start of the IWC SC review of the abundance estimate). Is Norway's unilateral action a rush to allow its whalers to kill the maximum number of whales before the IWC SC (which has been meeting 8–20 May 1995) provides a revised abundance estimate?

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Einstein's debt

SIR — In a recent lecture in the Netherlands on "What remains to be discovered?", your editor John Maddox said that Einstein should have acknowledged more frankly than he actually did his debt to Maxwell, Lorentz and Poincaré.

At Lorentz's funeral, Albert Einstein said: "To me he meant more than anybody I ever met in my life" (Leo Beek, *Dutch Pioneers of Science*, 1985).

Michaël W. J. van den Brink
The Hague, The Netherlands

Ebullient science adviser steps down

The retirement next week of Britain's Chief Science Adviser for the past five years will rob us of a forceful public servant and proselytizer of science at a time when there are too few.

SIR William Stewart, the British government's Chief Scientific Adviser, steps down next week after five years in which there has been a bigger upheaval in the public administration of British science than at any time in living memory. "Bill" Stewart (hardly anybody addresses him more formally) has been in the thick of the transformation. To all appearances, he has relished most of the past few years, but he has also agonized for much of the time. How well has he done?

Contrary to expectation, the British government has a penchant for appointing science advisers who are not conventional *apparatchiks*. They range from Solly Zuckerman (later Lord Zuckerman, who died last year), the zoologist turned polymath, who often talked as if his masters were his puppets, to patrician Lord Rothschild, who spoke of them alternatively as his servants and his idols: Rothschild revered Edward Heath, his prime minister.

Mrs Margaret Thatcher's advisers were, in succession, Sir Robin Nicholson (indistinguishable from a civil servant), who left to become research director at the glass-makers Pilkington, and Sir John Fairclough (more in his prime minister's swashbuckling style), who afterwards became something in the City of London. Stewart, by contrast, is unambiguously Mr John Major's man, even persuading his prime minister to preside at a press conference this week (see page 266) to bang the drum for science.

Stewart is unambiguously and ostentatiously a Scot. Born 60 years ago on the island of Islay (pronounced "eye-lah") off the West of Scotland, he wonders from time to time whether he may yet go back to "crofting" (which is the Scottish word for living honestly off the land). Like many bright lads from the Scottish islands, he graduated from the University of Glasgow (where he took his PhD as well). His professional line is the fixation of nitrogen. He joined the faculty at the University of Dundee at the age of 33, and was there as that university made its successful claim on public attention by building an excellent enterprise in molecular biology out of virtually nothing. His reward was a two year spell as secretary of the Agriculture and Food Research Council and then the move to Whitehall.

Stewart's strong suit is not guile (Zuckerman), combativeness (Fairclough) or magisterial certainty (Rothschild), but impatience; he is out to get things done.

He has had ample exercise of that ambition in the past five years, and ample experience of the frustration that follows constructive impatience.

Some credit Stewart with having pulled off the great coup (in 1992) of arranging the transfer of responsibility for the then-five research councils (now there are six) from the Department of Education and Science to the Cabinet Office, where they are now dependants of the Office of Science and Technology of which Stewart is effectively the chief executive officer (CEO). (In that nomenclature, the chairman is the political head, quaintly known as the Chancellor of the Duchy of Lancaster, now Mr David Hunt.)

Stewart declines sole credit for that radical administrative change, but acknowledges his support for the idea. The only way to put science and technology "at the heart of government", he says. Even critics of the change acknowledge that it has done that; their misgivings are that being at the heart of government makes the research councils vulnerable to whims of government departments fighting each other for influence.

That change had apparently been decided even before the election in April 1992, won by John Major against the odds offered by the opinion polls. The election brought Stewart not just a prime minister whom he recognized, but a talented minister (Mr William Waldegrave) who also had an interest in stirring the administrative pot. They (the two Williams) would have a period of public consultation and would then publish a White Paper (British governments' way of saying what it intends to do).

Stewart has been in overdrive ever since. Scots have the reputation of being taciturn, but Stewart has taken to public platforms like a professional. He has thrown his substantial frame about the United Kingdom (and elsewhere) indefatigably whenever there has been an audience of opinion-makers to address. At a private meeting at Cambridge a year ago, the news at breakfast was that there was "fog at Stornoway, and Sir William will be delayed". But everybody knew that he would not be delayed for long. He was there at lunch.

His big triumph is what is called "fore-sight", of which concept this journal has been steadfastly lukewarm. The idea is that a strategy for the formulation of priorities in research should evolve from a

process of continuing consultation among interested parties — researchers, industry and government. One objective has been to define priorities; another has been to persuade uncommunicative partners in what should be a common enterprise to talk to each other.

Judgement on the foresight exercise will no doubt be made by events that happen in the years ahead, but nobody can complain that the project has not been carried through energetically. There have been fifteen substantial reports on the subject in the past few weeks, together with a kind of wrap-up document last Monday. Goodness knows what mayhem that publishing programme has created. But the way in which governments elsewhere are sending emissaries to Britain to learn more of Stewart's concept of foresight is a striking measure of his influence.

In this as in the other events of the past five years, Stewart's outstanding feat has been his failure to make enemies. Part of the explanation has been his willingness to talk and even to listen to sometimes outlandish groups. (The pressure group called "Save British Science", which is not outlandish, has never previously been as closely heard.)

Another part of the explanation is Stewart's evident conviction that he has been doing the best he can for science. That is the Islay island lad's passion. Even working scientists who do not share his goals know a fellow whose heart is in the right place, and applaud him for it. That, they also remember, will have rubbed off on the civil servants with whom he has been mixing these past five years.

Being Scottish has also helped a lot. It is not just the accent (and, occasionally, the vocabulary), but the reminder that even Britain is changing quickly and that the Old Establishment may have used up its officer class. Stewart, a little subversively, is proud that his office sent the European Commission a paper saying what should be done about the Fourth Framework Programme that appears to have carried weight. Officials in Brussels are certainly respectful.

Stewart's capacity for ebullience has been his other strong suit. When things have been going well, he has cut the figure of a man so full of enthusiasm that he cannot possibly express it all in the time available. Public officials are rarely in that mould. It will be a pity if Stewart does indeed go back to crofting. **John Maddox**

Minute mantle melts

Rosamond J. Kinzler and Charles H. Langmuir

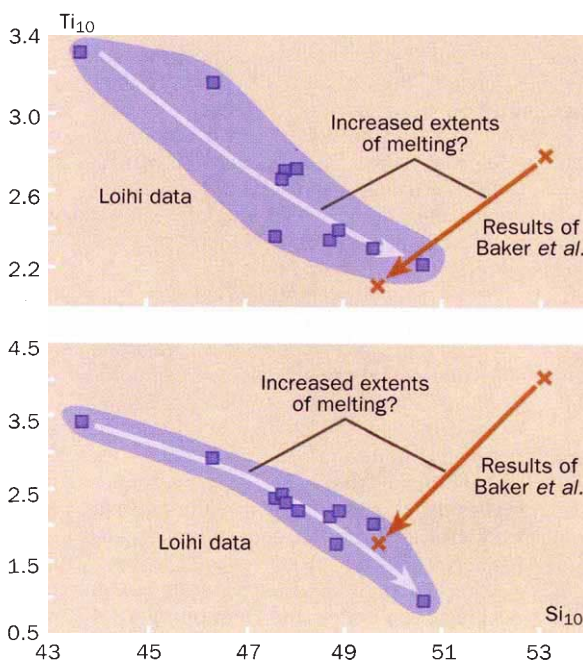
MELTING of the Earth's mantle has long been understood to be the primary driving force behind most volcanism on the Earth's surface. In recent years, better experimental data, physical models and observations have much improved our ability to model mantle melting and apply the models to physical and chemical observations. These results suggest that erupted magmas are pooled from a range of pressures and extents of melting that extend from very-low-degree melts at great depth to higher-degree melts (20 per cent or more) formed nearer the surface. There has been a nagging uncertainty, however, because direct experimental constraints on mantle melting have been restricted to melt percentages (F) that exceed 8–10 per cent; hence there has been controversy over the compositions of lower- F melts. Part of this uncertainty has been removed by new experimental data on low- F melts reported on page 308 of this issue by Baker, Hirschmann, Ghiorso and Stolper¹.

The low- F experimental data have additional significance because many elements, such as Th, U, K and Na, that convey information about the composition of the mantle source and about melt transport are most highly concentrated in low- F melts. Movement of minute quantities of such melts can change the concentrations of these elements substantially in the magma or the mantle.

There is compelling evidence that such low- F melts are present and important. The seismic low-velocity zone probably results from their presence². Most samples of mantle rock that come up as exotic xenoliths in volcanic rocks show evidence of 'metasomatism' by small-degree melts (see for example ref. 3), and the chemical compositions of many volcanic rocks appear to have been enriched or depleted by such melts^{4–6}. Therefore small-degree melts are an important key for our understanding of mantle processes, melt generation and the creation of mantle heterogeneity.

Although the compositions of small-degree melts have not been measured directly by experiment, they have been modelled using other relevant experimental data. With colleagues, we both pointed out^{7,8} that low-degree melts would be high in Na and low in Fe and Mg; calculations from ref. 8 match the new

experimental data well for these elements. Calculations in ref. 7 also predicted that low- F melts from spinel lherzolite would be richer in SiO_2 and Al_2O_3 and poorer in CaO than higher- F melts. These predictions are confirmed by Baker and co-workers' experiments. What was not predicted by earlier studies was the low TiO_2 at low melt fractions, the exceptionally high SiO_2 , and the decrease in the distribution coefficient for Fe–Mg exchange



Comparison of data from Loihi Seamount near Hawaii¹³ with the results from Baker *et al.*¹ in this issue. Rock data are normalized to 10 per cent MgO to remove the effects of low-pressure differentiation in magma chambers (for example, Si_{10} refers to Si at 10 per cent MgO). TiO_2 data for the experiments have been multiplied by 4.5 to permit comparison with the rocks. The low-Si, high-Na, high-Ti basalts are thought to be derived by the lowest extent of melting. This contrasts with the experiments of Baker *et al.* where the highest Si occurs at the lowest extent of melting. A likely cause for the discrepancy between the rocks and the experiments is the presence of volatiles during natural melting.

between olivine and liquid. These are important new aspects in the experimental results.

The new results account nicely for a puzzle that has emerged from the study of glass inclusions in mantle xenoliths. Recent work^{9,10} has shown that high-silica melt inclusions are widespread in mantle minerals, and the high SiO_2 of these melts did not resemble known compositions of melts from the mantle — until the high- SiO_2 , low- F melts of Baker *et al.* Although some of the melt inclusions have more extreme compositions than the experimental results, the experiments have no K_2O , and K_2O is likely to have an even

greater effect on melt compositions than Na_2O (refs 7, 8). Therefore even more extreme low- F compositions are possible.

A critical question will be to what extent these extreme melt compositions will modify existing conclusions from models of mantle melting. One can examine the chemical aspects of this question by including the high SiO_2 contents and high $D_{\text{Ti}}^{\text{cpx/melt}}$ of the low- F melts into an adiabatic melting model⁸. As the model already predicted the low- F results for Na, Fe and Mg, there is no effect for these elements. The effects on SiO_2 are also small (0.4–0.8 per cent) and may be within the resolution of the observations. TiO_2 is more

affected, particularly when melting starts at low pressures, and TiO_2 can be lowered in pooled melts by up to 20 per cent or so. The TiO_2 results may shed light on the generation of some high- Na_2O mid-ocean-ridge basalts, such as those from the Australian Antarctic Discordance in the Indian Ocean, that have anomalously low TiO_2 contents.

The strikingly low TiO_2 abundances observed in the low- F experimental melts of Baker *et al.* reflect an increase in the partition coefficient for Ti between clinopyroxene and melt ($D_{\text{Ti}}^{\text{cpx/melt}}$) as F diminishes. This change has been anticipated by earlier studies of trace element partitioning between pyroxene and melt¹¹ which demonstrated that $D_{\text{Ti}}^{\text{cpx/melt}}$ correlates with the abundance of Al in the clinopyroxene and coexisting melt. If Al abundance in the clinopyroxene is a principal control, then one can infer that near a pressure of 1 gigapascal, $D_{\text{Ti}}^{\text{cpx/melt}}$ may be near a maximum value. At greater pressures, particularly at pressures where garnet is present, Al abundances in coexisting melts and clinopyroxenes would be less, and $D_{\text{Ti}}^{\text{cpx/melt}}$ may be lower as well.

The data obtained by Baker *et al.* are an important step forward. But consideration of other data shows that there is still much to learn about low- F melts. The classic example of low- F melts in igneous petrology has been the alkaline rocks that are found at all volcanic ocean islands and on continents. The figure shows data from Hawaiian volcanics that extend from very-low-degree melts (nephelinites) up to the tholeiites thought to represent large extents of melting. The lower-degree melts have low SiO_2 , very high TiO_2 , high FeO and high Na_2O — very different from the results of Baker *et al.* In fact, the trend of the chemical data in the figure is orthogonal to the trend defined by the new experimental melt compositions with increasing F . How could these natural data

apparently be so inconsistent with the new experiments?

The answer most probably lies in the role played by volatiles in the generation of many low-*F* melts in the Earth's mantle. Water and carbon dioxide are thought to have considerable influence on both the physical properties and compositions of melts, and volatiles are very important for the petrogenesis of alkaline igneous rocks such as those shown in the figure¹². It seems likely, for example, that the major element compositions of low-*F* alkaline

melts are controlled by the CO₂ abundances of the mantle source. Clearly a more complete understanding of low-*F* melts will require the addition of volatiles and K₂O to future experiments. So the results of Baker *et al.* provide new constraints on low-*F* melts — and there are many avenues still to explore. □

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1. Baker, M. B., Hirschmann, M. M., Ghiorso, M. S. & Stolper, E. M. *Nature* **375**, 308–311 (1995).
2. Forsyth, D. in *Mantle Flow and Melt Generation at Mid-Ocean Ridges*, *Geophys. Monogr.* 71 (eds Morgan, J. P., Blackman, D. K. & Sinton, J. M.) 1–66 (1992).
3. *Mantle Metasomatism* (eds Menzies, M. A. & Hawkesworth, C. J.) (Academic, Orlando, 1987).
4. Galer, S. J. G. & O'Nions, R. K. *Chem. Geol.* **56**, 45 (1986).
5. McKenzie, D. *Earth planet. Sci. Lett.* **74**, 81–91 (1985).
6. Schilling, J.-G., Kingsley, R. H. & Devine, J. D. *J. geophys. Res.* **87**, 5593–5610 (1980).
7. Kinzler, R. J. & Grove, T. L. *J. geophys. Res.* **97**, 6885–6906 (1992).
8. Langmuir, C. H., Klein, E. M. & Plank, T. in *Mantle Flow and Melt Generation at Mid-Ocean Ridges*, *Geophys. Monogr.* 71 (eds Morgan, J. P., Blackman, D. K. & Sinton, J. M.) 183–280 (1992).
9. Draper, D. S. *Geology* **100**, 766–776 (1992).
10. Schiano, P. & Clacchiatti, R. *Nature* **368**, 621–624 (1994).
11. Gailanhan, W. E. & Neilsen, R. L. *Geochim. cosmochim. Acta* **56**, 2387–2404 (1992).
12. Wyllie, P. J. *Earth planet. Sci. Lett.* **82**, 391–397 (1987).
13. Frey, F. A. *et al.* *Earth planet. Sci. Lett.* **66**, 337–347 (1983).

VIROLOGY

When it's better to lie low

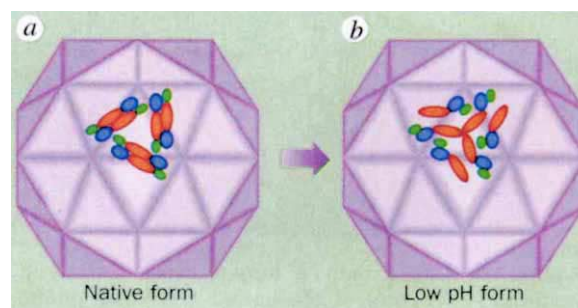
Richard J. Kuhn and Michael G. Rossmann

IN 1981, Wiley and co-workers¹ reported the atomic structure of haemagglutinin, a spike-like surface glycoprotein of influenza virus. This and subsequent work led to an explanation of the occurrence of flu pandemics² and determination of the site of binding to the sialic acid receptor³, and has given insight into the extensive conformational changes that occur upon viral fusion with the membrane of the cell under attack⁴. Now, on page 291 of this issue⁵, Rey *et al.* describe the structure of the external glycoprotein of tick-borne encephalitis (TBE) virus and it promises to be of equal scientific and medical significance. The structure determination itself was a heroic effort, involving the application of many of the techniques learned by the structural community over the past 30 years.

TBE belongs to the flavivirus family, which includes many members of medical importance such as yellow fever, dengue, Japanese encephalitis and the more distantly related hepatitis C virus. These are a group of enveloped, positive-strand RNA viruses with an external diameter of around 500 Å. The flavivirus envelope contains two transmembrane proteins: the small M protein and the major surface glycoprotein E. This second envelope protein is responsible for receptor recognition and membrane fusion, and is the primary site for neutralizing antibody induction, properties that it shares with influenza haemagglutinin.

Rey *et al.* released the amino-terminal 395 amino acids of the virion envelope

protein by cleavage with trypsin⁶. This soluble envelope fragment (sE) was crystallized in a dimeric form, probably reflecting the wild-type structure at physiological pH (ref. 6). The dimer structure has a symmetry axis that is perpendicular to its length, in contrast to the influenza haemagglutinin spike structure which has



The conformational change that might occur in the E glycoprotein upon exposure to low pH during the membrane fusion process. *a*, The infectious virion at neutral pH showing E dimers as found by Heinz *et al.*⁶ in a hypothetical *T*=3 surface lattice. Domain I is shown in blue, the dimerization domain II in red, and domain III in green. *b*, The low pH form in which domain II has rearranged by a clockwise rotation of 30° to form trimers.

its symmetry axis parallel to its length. Furthermore the dimer has the shape of a slightly bent, 150-Å-long rod. The carboxy termini point towards the inner concave side, whereas the two glycosylation sites in the dimer are on the external convex side, as also are loops that vary in sequence between different flaviviruses. Rey *et al.* therefore propose that, unlike

the spikes seen on many viruses, including influenza, HIV and Sindbis, the flavivirus envelope protein is lying down (see figure), perhaps with its two-fold axis coincident with symmetrical features on the internal nucleocapsid as seen in the alphaviruses⁷.

The monomer of sE has a structure with three distinct domains (see Fig. 1, page 293). The central domain (I) is a nine-stranded, antiparallel β-barrel. Many viral coat proteins have an antiparallel β-barrel structure, but the topology in TBE does not resemble this frequently found fold. Instead the central domain is composed of three separated segments with the two intermediate loops making up the dimerization domain (II). The carboxy-terminal 93 residues form an immunoglobulin(Ig)-like carboxy terminal domain (III). The unique glycosylation site is on domain I, covering a highly conserved hydrophobic loop of domain II from the adjacent monomer that fits into a groove between domains I and III. Distinct antigenic sites have been mapped to all three domains, although only the epitopes on domain II cross-react between flaviviruses.

In mosquito-borne flaviviruses, the distal face of domain III contains four more amino acids than that of TBE and other tick-borne flaviviruses, implying that these residues are likely to be involved in host differentiation. Many of the mosquito-borne flaviviruses contain the RGD sequence motif that is recognized by integrins, which are common ligands for Ig adhesion molecules. The insertion of the RGD sequence in one of the loops of the Ig-like domain is identical to its

location in some fibronectin type III domains⁸, suggesting that it has a functional role.

The existence of an Ig-like domain in a virus structural protein hints at an interesting strategy in virus-cell recognition. Many viruses use cellular adhesion molecules as receptors for cell entry. The role of Ig superfamily members as viral receptors has been well established in numerous systems, including CD4 for HIV, ICAM-1 for rhinovirus and CEA for mouse hepatitis virus. The TBE structure demonstrates that the Ig domain, commonly found on cellular receptors, can be a viral antireceptor as well. Flaviviruses, however, may not be unique in this approach to cellular recognition — another structure determination of an insect virus also points to an Ig domain as the site of receptor attachment (J. E. Johnson, personal communication).

Like influenza viruses, the flaviviruses

are thought to undergo receptor-mediated endocytosis. Under conditions of low pH, the E dimer reorganizes itself to form a trimer⁹ and activates the fusion process. The residues responsible for fusion, the so-called fusion domain, map to a highly conserved hydrophobic loop in domain II which has been implicated in the fusogenic activity of the virus¹⁰. Exactly how the E dimer reorganizes to form a trimer remains obscure, but from data derived from antibody probes it seems that there is a significant reorganization of domain II following the conformational change. The soluble E dimer used by Rey *et al.* fails to undergo the conformational change, suggesting that the additional E sequences, or perhaps the M protein, may be required for inducing the remarkable change necessary to bring about membrane fusion.

Exactly how the E dimer is arranged on

the virus surface, and its possible connections to the M protein and to the inner nucleocapsid protein, are also unclear. The size of sE is too big for it to have $T=4$ symmetry but it might be consistent with $T=3$ symmetry. For example, if the glycoprotein dimer axes were placed coincident with the icosahedral and quasi two-fold axes, a rearrangement of domain II induced by changes in pH would bring these domains together to form trimers and abrogate the dimer contacts (see figure). At the same time this would expose the fusogenic peptide previously hidden in a pocket under the attached carbohydrate. Future studies of TBE could include cryoelectron microscopy analysis which would be useful to identify the symmetry of the virion.

The structure of the major envelope protein of a flavivirus provides us with a

multitude of new questions. It also shows, once again, the resourcefulness of viruses when attacking their cellular prey. □

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1. Wilson, I., Skehel, J. & Wiley, D. *Nature* **289**, 366–373 (1981).
2. Wilson, I. A., Wiley, D. C. & Skehel, J. J. *Nature* **289**, 373–378 (1981).
3. Weis, W. *et al.* *Nature* **333**, 426–431 (1988).
4. Bullough, P. A., Hughson, F. M., Skehel, J. J. & Wiley, D. C. *Nature* **371**, 37–43 (1994).
5. Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C. & Harrison, S. C. *Nature* **375**, 291–298 (1995).
6. Heinz, F. X. *et al.* *J. Virol.* **65**, 5579–5583 (1991).
7. Cheng, R. H. *et al.* *Cell* **80**, 621–630 (1995).
8. Leahy, D. J., Hendrickson, W. A., Aukhil, I. & Erickson, H. P. *Science* **258**, 987–991 (1992).
9. Allison, S. L. *et al.* *J. Virol.* **69**, 695–700 (1995).
10. Roehrig, J. T., Hunt, A. R., Johnson, A. J. & Hawkes, R. A. *Virology* **171**, 49–60 (1989).

PARASITISM

Mycological mystery tour

FROM the mossy depths of the spruce forests of the Olympic peninsula in Washington state comes an eldritch tale. For within these ancient forests are mushrooms growing on the living tissues of yet other mushrooms: the hosts may remain fertile but become deformed almost beyond recognition.

The pictured specimen of a *Galerina* species, from which grow several fruiting bodies of the parasitic *Squamanita*, is unusual in that it still looks like a mushroom (for scale, the cap is 6 mm across). It was this specimen, in part, which enabled S. A. Redhead and colleagues to resolve a thorny taxonomic tangle which has long been the bane of mycologists (*Canadian Journal of Botany* **72**, 1812–1824; 1994). The swollen, oddly coloured basal 'protocarpic tubers' from which *Squamanita* fruiting bodies were seen to emerge were so distorted that they were long thought to be parts of *Squamanita* itself, rather than the tissues of hosts made shapeless by possession. Redhead and colleagues now number *Squamanita* as one of a small number of fungal genera which are obligate parasites of other fungi.

The story begins as long ago as 1918, in the Netherlands. Botanists were struck by the unexpected appearance, in several locations, of a remarkable new species of fungus notable for the odour and colour of grapes, and its curiously swollen base. A second species, odourless but bright yellow — and again with a swollen, bulb-like base — was found in Switzerland in the mid-1930s. The botanist L. Schreier, who described this species, nurtured the isolated bulbs in a darkened

cellar and showed that they sprouted mushrooms in the absence of a supporting mycelium. It was this second occurrence which inspired the creation of the genus *Squamanita*, based largely on the presence of the basal bulb.

Several decades of mycological disquiet set in, as collectors and researchers suspected that *Squamanita* was



S. A. Redhead

more than a fungal curio. Persistent reports of the varied character of the 'protocarpic tubers' suggested that they were less constant than taxonomic propriety demanded. More worrying were the noted resemblances between these tubers and the body parts of other fungi. One such was the agaric *Cystoderma paradoxum*, the stem of which seemed to be made of two different tissues, separated in the middle by an annular constriction. The tissues of the thicker bottom half were typical of its genus, but these tissues graded upwards into the thinner stalk and cap of a *Squamanita*. This unwholesome chimera was removed to the genus *Squamanita*, the peculiar *Cystoderma*-like base being taken as just another

example of a *Squamanita* protocarpic tuber.

It was then that suspicions of parasitism arose, as examples of this odd species were collected from within colonies of otherwise normal examples of *Cystoderma*. "It seems as if another agaric has been grafted on to the stem of *Cystoderma amianthinum*", observed one collector. Mycologists began to view *Squamanita* as a chameleon, parasitizing *Cystoderma* to the extent of perverting the tissues destined to be the upper stalk and cap of the mushroom to its own image — giving the appearance of a *Squamanita* growing from a tuber with an uncanny resemblance to *Cystoderma*.

Support for this idea came from Japan, with the discovery in 1990 of an example of *Squamanita* with a protocarpic tuber bearing characteristic features of another fungus, *Phaerolepiota aurea*. The clear-cut parasitism of *Squamanita* on specimens of *Galerina* from the Olympic peninsula now unequivocally exposes the relationship between host and unwelcome guest.

The solution raises another problem. The genus *Squamanita* is traditionally defined on the presence of the basal tuber. But this is invalid if these tubers are really the shattered wrecks of other fungi. Redhead and colleagues pick their way out of the impasse to justify the existence of *Squamanita* as a cadre of obligate parasites of fungi, and then review all other similar instances. The result is an enjoyable snapshot of some 80 years of thinking about the phenomenon.

Henry Gee

Growth through learning

Michael P. Stryker

CONNECTIONS between neurons in the brain are thought to be made, broken and adjusted in strength both during learning¹ in adult life and during development^{2,3}. Specific cellular changes called long-term potentiation and depression (LTP and LTD) seem to be responsible, at least in large part, for an important form of learning^{4,5}. Are similar mechanisms involved in the refinement of neural connections in development? Papers on pages 325 and 328 of this issue^{6,7} provide correlative evidence that they are.

Unlike a computer, in which a circuit diagram specifies the connections in advance, the brain must wire itself. At least in primary sensory cortical areas, the brain refines its connections to a state of high precision during a critical period in early life, in a form of unsupervised learning⁸. During this critical period, but not before or after, synaptic connections between neurons are highly plastic and respond dramatically to alterations in the pattern of neural activity⁹⁻¹¹.

Cellular mechanisms of neural plasticity have been worked out in greatest detail in the hippocampus^{5,12}, where the study of LTP and LTD has become a growth industry. The use of the slice preparation allows a specific class of synapse to be manipulated and studied directly *in vitro*, and a great deal has been learned about the cellular events that underlie the changes in the strength of these synapses. The question, again, is whether plasticity in cortical synapses during the critical period is carried out by the same cellular

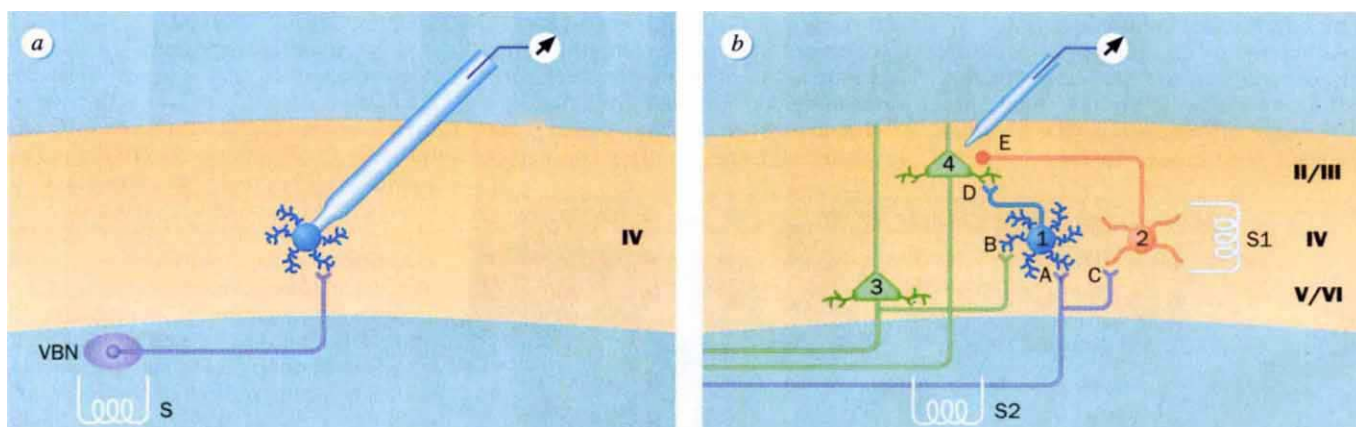
machinery responsible for LTP in the hippocampus. Many features of the synapses are similar, and the same neurotransmitters and receptors seem to be involved. If we were confident that the mechanisms were indeed the same, then work on cortical development could piggy-back on the hippocampal story.

One way of tackling the question is to look for correlations between the occurrence of LTP and manipulations known to affect developmental cortical plasticity *in vivo*. The most prominent feature of such plasticity is the critical period. Crair and Malenka⁶ and Kirkwood *et al.*⁷ take advantage of it by comparing LTP in slices from animals of different ages, and both find forms of LTP during the critical period that are smaller or absent thereafter. In addition, Kirkwood *et al.* raise animals in darkness, which appears to extend the critical period *in vivo*¹³, and they find that susceptibility to one sort of LTP is also extended. The findings are consistent with the notion that LTP *in vitro* involves the same mechanisms that organize neuronal connections in development.

An understanding in cellular and molecular terms of the mechanisms responsible for properly connecting up neurons in the cortex would not only be tremendously satisfying on intellectual grounds, it would also have valuable implications for further experiments and for attempts at therapy. The power of studies of LTP *in vitro* is that, unlike most experiments that can be carried out on develop-

ment *in vivo*, the particular synapses that change can be identified, and their properties can be made susceptible to pharmacological and biochemical analysis. Crair and Malenka's study on somatic sensory cortex realizes this promise. Their slice preparation allows them to find the synapse (at the earliest or input stage of cortical processing) at which changes take place, and further cellular studies of this synapse in this system can only enrich our understanding of the mechanisms that control cortical plasticity in development.

Kirkwood and colleagues' findings on visual cortex present a more complicated picture. These authors study the aggregate activity produced in the upper layers (which constitute the second stage of cortical processing) by the whole visual cortical circuit, monitored by extracellular recordings. They find that one form of LTP, evoked by stimulation of layer IV (electrode S1 in the figure), is of similar magnitude no matter what the age of the slice; while a second form of LTP, evoked by stimulation of white matter (electrode S2), disappears after the critical period (confirming earlier findings). Because of the complex nature of the circuitry activated by S2, the change in synaptic efficacy responsible for the plasticity during the critical period could occur at any of synapses A-E shown in the figure. The disappearance of plasticity after the end of the critical period could equally result not from a loss of plasticity at the originally plastic site but from compensatory changes at any of these loci. Indeed, the authors' hypothesis to explain the end of the plasticity *in vitro* is an increase in inhibition produced by cells such as cell 2 in the figure, rather than some decrement in a synaptic mechanism of plasticity. This



a, The thalamocortical slice experiment of Crair and Malenka⁶, showing the synapse from the thalamic relay neuron (in ventrobasal nucleus, VBN, near stimulating electrode S) onto the layer IV cortical that becomes stronger after LTP during the first week but not later in life. Whole-cell, patch-electrode records voltage response to and current through this particular synapse. b, Experimental arrangements of Kirkwood *et al.*⁷, showing some of the synapses (A, B, C, D, E) at which long-term changes may have taken place after tetanic stimulation at stimulating electrodes S1 or S2. Neurons 1, 3 and 4 are excitatory, and neuron 2 is inhibitory. S2 will activate all the neurons and synapses

indicated. S1 will activate all the synapses illustrated and will activate at least neurons 1 and 2 directly and neuron 3 synaptically. Extracellular electrode records small voltage changes in the fluid outside the cells due to aggregate activity at many synapses like those illustrated. Increases in these voltage changes are interpreted as increases in the synaptic activation of cells such as neuron 3, produced by either an increase in the efficacy or activity of excitatory synapses (D for example) or a decrease in the efficacy or activity of inhibitory synapses (E for example). Selected cortical layers are indicated by roman numerals.

explanation may well be correct, but it is not clear what we have gained in simplicity by taking the system *in vitro*. It is certainly not yet clear from these findings whether changes in the actual cellular mechanisms of plasticity at the input synapse could account for the end of the critical period in rodent visual cortex, as Crair and Malenka have now shown evidence for in somatic sensory cortex.

'Long-term potentiation' is often taken to refer to a cellular mechanism. But it actually denotes a wide variety of phenomena, for only a few of which is there

any understanding in cellular terms. One hopes that the unity of biology will triumph in the end and that common mechanisms will be found to operate in all neocortical areas as well as in hippocampus. For now, however cognizant we may be of the promise of a cellular understanding of visual cortical plasticity, we must continue to seek it. □

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1. Squire, L. R. & Lindenlaub, E. (eds) *The Biology of Memory Symp. Medica Hoechst* 23 (Schattauer, Stuttgart, 1990).
2. Goodman, C. S. & Shatz, C. J. *Cell* **72** Suppl. 77–98 (1993).
3. O'Leary, D. D., Ruff, N. L. & Dyck, R. H. *Curr. Opin. Neurobiol.* **4**, 535–544 (1994).
4. Morris, R. G. *Cold Spring Harb. Symp. quant. Biol.* **55**, 161–173 (1990).
5. Bliss, T. V. & Collingridge, G. L. *Nature* **361**, 31 (1993).
6. Crair, M. C. & Malenka, R. C. *Nature* **375**, 325–328 (1995).
7. Kirkwood, A., Lee, H.-K. & Bear, M. F. *Nature* **375**, 328–331 (1995).
8. Miller, K. D. *Prog. Brain Res.* **102**, 303–318 (1994).
9. Wiesel, T. N. *Nature* **299**, 583–591 (1982).
10. Antonini, A. & Stryker, M. P. *Science* **260**, 1819–1821 (1993).
11. Fox, K. & Zahs, K. *Curr. Opin. Neurobiol.* **4**, 112–119 (1994).
12. Bear, M. F. & Malenka, R. C. *Curr. Opin. Neurobiol.* **4**, 389–399 (1994).
13. Fagioli, M., Pizzorusso, T., Berardi, N., Domenici, L. & Maffei, L. *Vision Res.* **34**, 709–720 (1994).

PHOTONICS

Minding the gap

John D. Joannopoulos

IMAGINE a microscopic metropolis, at micrometre length scales, with buildings that can house bundles of light, and highways and bridges that can guide light along narrow channels and around tight corners. Such are the dreams of researchers in the optoelectronics industry. And during the March meeting of the American Physical Society (San Jose, 20–24 March 1995), work presented at a symposium provided hope that such dreams may soon become a reality. The key is in the use of a new class of materials called photonic crystals.

A photonic crystal is a perfectly periodic and intricate dielectric structure specifically designed so that light in a given range of frequencies (the photonic bandgap) is forbidden to exist in its in-

terior. Forbidden, that is, unless there is a defect within the otherwise perfect crystal. A defect acts like an internal surface within which a bundle of light could be trapped. This new ability to localize light could be used to design elements of novel microphotonic devices in optoelectronic integrated circuits.

The first investigations of photonic crystals were carried out at millimetre wavelengths in the early 1990s. As the size of a photonic crystal scales with the wavelength of the light, experimental investigations at long wavelengths permitted fabrication of photonic crystal structures using conventional machine tools.

But how can one begin to fabricate such intricate structures for sizes approaching

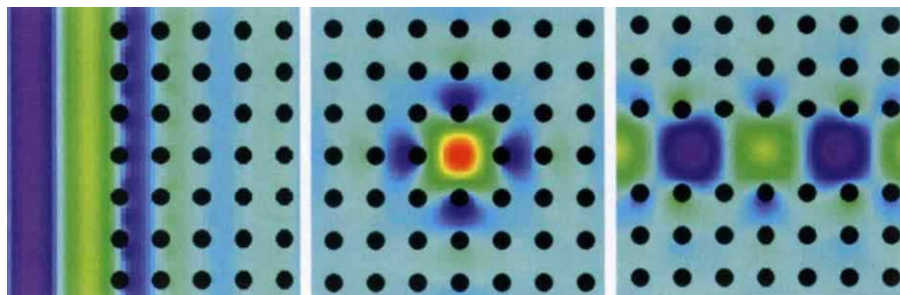
micrometre length scales? One proposed solution is based on micromachining silicon wafers which have been oriented along (110) (Ekmel Özbay, Ames Laboratory, Iowa State Univ.). This fabrication technique takes advantage of a new dielectric-rod based design which can be constructed by stacking wafers in a specific order. A variety of photonic crystal structures have been made in this fashion, some with lattice constants as small as 600 micrometres. These are the smallest photonic crystals yet fabricated, and Özbay believes he can eventually reach lattice constants of 150 micrometres with his technique.

But even if photonic crystals can be fabricated at microscopic length scales, what can we do with them? One novel microphotonic device element has been devised by introducing a line defect in an otherwise perfect photonic crystal, so that this defect would act as a waveguide (Pierre Villeneuve, Massachusetts Inst. Technol.). This approach was then used to design high-efficiency waveguide bends with a radius of curvature comparable to the wavelength of the light. Another new design was a class of resonant microcavities integrated directly in the plane of the substrate (Villeneuve). These coplanar microcavities are designed as microscopic bridges which allow for efficient coupling into other components of optoelectronic integrated circuits, as well as direct high-speed modulation and single-mode output.

The current interest in photonic crystals, however, is by no means limited to micrometre length scales. At the same symposium, an exciting design was unveiled for a metallodielectric photonic crystal structure with a large gap at microwave frequencies (Elliott Brown, Massachusetts Inst. Technol.). This crystal is a periodic arrangement of metallic beads embedded in a uniform dielectric medium. It is configured so as to have no surface modes and was used as a substrate for an array of phased directional planar antennae. The large gap and lack of surface modes eliminate cross-talk between the antennae and allows them to radiate most of their power efficiently into free space above the substrate.

Five years ago, the Yablonovitch concept of a photonic crystal appeared to many casual observers as fanciful speculation. The work presented at this year's meeting, together with recent achievements from groups around the world, clearly establishes the legitimacy and promise of this new field. It may well be that, as yet, we have only seen the tip of the iceberg. □

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A photonic crystal, depicted as a simple square lattice of dielectric rods in air, is shown in three cases. In the left panel, a light wave is incident from the left. Its frequency lies in the photonic bandgap, so the wave is forbidden to propagate inside the crystal. Removal of one rod, as in the centre panel, creates a defect which can trap a bundle of light within its interior in a very localized state. Such a defect could act as a cavity which contains only one mode. Removal of a series of rods, as in the right panel, can trap light along a line reminiscent of a traditional dielectric waveguide. An important difference, however, is that the photonic crystal allows the light to travel in air, thereby reducing losses incurred in the dielectric.

Patterning goes Sonic

Jean-Paul Concordet and Philip Ingham

GRADIENTS of morphogens have long been seen as attractive solutions to the problem of how cells acquire different identities during embryonic development. Examples have been in short supply, however, apart from in *Drosophila*. Activin, a member of the transforming growth factor- β family, can induce transcription of different genes in a concentration-dependent manner in *Xenopus* embryonic explants^{1,2}. But its involvement in normal embryonic development remains controversial — activin expression is not consistent with its postulated function in *Xenopus* development and, at least in the mouse, absence of all known activin genes during embryogenesis has little discernible effect on the specification of cell fate³.

On page 322 of this issue⁴, Martí *et al.* describe another example of a secreted protein that activates transcription of different genes in a dose-dependent manner. In this case, however, the protein, Sonic hedgehog (Shh), is expressed at the right time and in the right places to be a bona fide morphogen.

Sonic hedgehog is one of several vertebrate homologues of the *Drosophila* gene *hedgehog* (*hh*), which has a key part in patterning the segments of the developing fly. Like *hh*, expression of *Shh* is highly localized and at the early stages of embryogenesis is restricted largely to the notochord and floor plate^{5–8}, two midline structures implicated in inductive interactions that control the patterning of the neural tube⁹. Cells along the ventral midline of the neural tube are induced to differentiate floor plate by the underlying notochord, a process that can be reproduced *in vitro* but only if the inducing and responding tissues are in direct contact. By contrast, cells lateral to the floor plate are induced to differentiate into motor neurons by notochord and floor plate, a process that can occur *in vitro* in the absence of contact between the tissues.

This qualitative difference between the two inductive interactions implies that they may be mediated by distinct signals. Evidence that *Shh* encodes the floor-plate-inducing signal comes from the findings that ectopic expression of Shh *in vivo* leads to the activation of floor-plate markers in inappropriate regions of the neural tube^{5,6}, and that COS cells expressing Shh can induce floor-plate differentiation in neural tube explants⁸. Whether or not *Shh* could also encode the motor-neuron inducer is less easy to discern, in that such induction could be a secondary consequence of the induction of floor plate⁸.

Studies of the *hh* mutant phenotypes have been interpreted in terms of hedgehog protein (Hh) mediating two distinct

signalling activities, one short range and one long range. A possible molecular basis for such a functional dichotomy comes from the discovery that Hh undergoes autoproteolytic cleavage to generate two distinct subspecies^{10,11}. The cleavage generates two fragments of unequal size, the smaller of which is amino-terminally derived and is the part of the protein that is most highly conserved between different species. The carboxy-terminal fragment is more variable, although certain regions, including domains required for autoproteolysis, are highly conserved. Intriguingly, antibodies specific for the amino-terminal fragment detect protein mostly around the periphery of expressing cells, whereas the carboxy-terminal fragment appears to diffuse away from the expressing cells. The second fragment, then, has the properties expected of a long-range signalling molecule, while the first displays characteristics consistent with a short-range or contact-dependent signal. Similar studies of Shh show that it is processed in an essentially identical manner^{11,12}.

Martí *et al.*⁴, and also Roelink *et al.* in a paper published earlier this month in *Cell*¹³, now provide unequivocal evidence that all of the signalling activity of Shh in the neural tube resides exclusively within its amino-terminal fragment (ShhN). Using either recombinant proteins^{4,13} or tissue-culture medium conditioned by cells expressing deletion constructs of the Shh complementary DNA¹³, they show that ShhN, but not the carboxy-terminal fragment (ShhC), is effective at inducing floor-plate and motor-neuron differentiation *in vitro*. Both groups also demonstrate that induction of the two cell types can be separated using different concentrations of ShhN. So the distinction between contact-dependent and diffusible signalling activities seems simply to reflect the fact that different cellular responses are induced by different threshold levels of ShhN activity.

The analysis of Roelink *et al.* was facilitated by the discovery that ShhN generated artificially by expressing a truncated Shh cDNA is readily secreted by cells in tissue culture^{11,12}. Apart from the implication that autoproteolytic cleavage of the full-length protein results in a modification of ShhN that promotes its association with the cell surface, this finding has the practical consequence that cells expressing the construct produce conditioned medium with significant inducing activity. Medium containing ShhN at concentrations of 5 nM or higher can induce expression of the floor-plate marker HNF3 β in neural-plate explants, whereas concentra-

tions of 1.25 nM induce expression of Islet-1, a marker that is specific for motor neurons. By contrast, Martí *et al.* find that transcription of floor-plate marker genes could be induced only when neural plate cells are presented with ShhN protein coupled to nickel beads, whereas — like Roelink *et al.* — they find that medium containing soluble ShhN can induce motor neurons.

Additionally, Martí *et al.* show that motor-neuron induction by the notochord can be inhibited by antibodies against ShhN which, they suggest, is an indication that ShhN is not only sufficient but necessary for motor-neuron induction. However, inhibition of motor-neuron induction may well have been secondary to inhibition of floor-plate induction (which was not examined in these studies). Other experiments testing the consequences of inactivating Shh are clearly needed.

Shh also appears to mediate a second, contact-independent inducing activity of the notochord, the induction of sclerotome differentiation in somites^{7,14}. In new studies by Fan *et al.*, also just published in *Cell*¹⁵, this second, contact-independent inducing activity of Shh is also shown to reside within ShhN. At concentrations similar to those sufficient for motor-neuron induction in the absence of floor plate, it induces expression of a sclerotome-specific marker in presomitic mesoderm explants.

In all, the implication is that the preferential retention of ShhN on the surface of cells from which it is secreted creates a gradient of ShhN. The consequence, it seems, is that cells in direct contact with the notochord are induced to form floor plate, whereas more distant cells experience levels of ShhN compatible with motor-neuron differentiation in the neural plate or sclerotome differentiation in the presomitic mesoderm.

Is this enough to explain cell patterning in the ventral neural tube? *In vivo*, ShhN can only be detected at the surface of the cells expressing *Shh*¹², so the difference in ShhN concentrations that are sufficient

- Green, J. B. A. & Smith, J. C. *Cell* **71**, 731–739 (1992).
- Gurdon, J. B., Harger, P., Mitchell, A. & Lemaire, P. *Nature* **371**, 487–492 (1994).
- Smith, J. C. *Nature* **374**, 311–312 (1995).
- Martí, E., Bumcrot, D. A., Takada, R. & McMahon, A. P. *Nature* **375**, 322–325 (1995).
- Echelard, Y. *et al.* *Cell* **75**, 1417–1430 (1993).
- Krauss, S., Concordet, J.-P. & Ingham, P. W. *Cell* **75**, 1431–1444 (1993).
- Johnson, R. L., Laufer, E., Riddle, R. D. & Tabin, C. *Cell* **79**, 1165–1173 (1994).
- Roelink, H. *et al.* *Cell* **76**, 761–775 (1994).
- Smith, J. C. *Curr. Biol.* **3**, 582–585 (1993).
- Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A. & Beachy, P. A. *Science* **266**, 1528–1537 (1994).
- Porter, J. A. *et al.* *Nature* **374**, 363–366 (1995).
- Bumcrot, D. A., Takada, R. & McMahon, A. P. *Molec. cell. Biol.* **15**, 2294–2303 (1995).
- Roelink, H. *et al.* *Cell* **81**, 445–455 (1995).
- Fan, C.-M. & Tessier-Lavigne, M. *Cell* **79**, 1175–1186 (1994).
- Fan, C.-M. *et al.* *Cell* **81**, 457–465 (1995).
- Kennedy, T. E., Serafini, T., Delattre, J. R. & Tessier-Lavigne, M. *Cell* **78**, 425–435 (1995).
- Blair, S. *Nature* **373**, 656–657 (1995).

for floor-plate and motor-neuron induction must be greater than *in vitro*. Perhaps other factors secreted from the notochord cooperate with ShhN in motor-neuron and sclerotome induction. Netrin 1 might be one, because like Shh it is expressed from both notochord and floor plate¹⁶.

High levels of ShhN are also found at the surface of the floor plate, leaving the question of why all cells next to the floor plate don't in turn become floor plate. Is it just a matter of timing, with motor-neuron commitment preceding ShhN expression at high levels from the floor plate, or are there inhibitory pathways at work? Experiments where neural-plate explants are successively exposed to low (motor-neuron inducing) and then high (floor-plate inducing) concentrations of ShhN might help in addressing this issue. Furthermore, motor-neuron differentiation can be detected only after several cell divisions, so it remains to be seen whether ShhN exerts its effect only by inducing

these divisions, or whether it does indeed affect their outcome and the engagement into motor-neuronal differentiation.

One can expect further progress from studies of the Hh signalling pathway and its likely association with amplification and inhibition pathways. Indeed, Fan *et al.*¹⁵ show that one component known to play a role in the fly¹⁷, protein kinase A, is also involved in sclerotome induction by Shh, indicating that these networks of signalling pathways are highly conserved. Such molecular approaches, used in both vertebrate and invertebrate model organisms, will continue to push back our ignorance of the mechanisms involved in pattern formation during vertebrate development. □

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EVOLUTIONARY BIOLOGY

Honesty in sexual selection

William J. Sutherland and John D. Reynolds

In their paper on page 311 of this issue¹, Gustafsson and colleagues reveal links between sexual selection and life-history traits. Their study of collared flycatchers (*Ficedula albicollis*) examined the size of the white patch on the forehead of males (see photograph). This modest feature proves to be a remarkably honest indicator of the males' past reproductive efforts and of the quality of their sons' upbringing.

Sexual selection is a topic with a long history, for it was Charles Darwin² who pointed out that many of nature's extravagances, such as bright colours, loud songs and ornamental plumages, might have evolved because they help their bearers attract mates or intimidate rivals. But why should animals be impressed by each others' displays? An important theory in sexual selection^{3,4} views display traits as reliable signals of quality — if the traits are costly to maintain, only individuals in good condition can afford the more elaborate displays and hence may be better mates or more formidable opponents. Such costs lie at the heart of an independent theory of life-history evolution^{5,6}, which predicts a trade-off between current reproductive effort and the ability to invest in future reproduction. It is these two theories that Gustafsson *et al.* have connected.

The forehead patch of male collared flycatchers is acquired during a spring moult on the African wintering grounds. When males reach the breeding grounds, those with a larger patch are more success-

ful in aggressive encounters over nest boxes and produce slightly more offspring that survive to join the breeding population. The forehead patch may thus act as a signal of status that is reliable because of the high social cost to males in poor condition that try to cheat^{7,8}.

Gustafsson and colleagues reasoned that if such sexual signals do indicate physical condition, and if there are significant costs of reproduction, then males that are induced to increase their reproductive effort should be in worse physical condi-

tion the following year. This should then be reflected by the development of a smaller forehead patch the following spring.

An elegant series of experiments supported this idea. The authors manipulated male reproductive effort by adding or removing one or two eggs from nests. As predicted, when the birds returned the following year those males which had been induced to care for enlarged clutches had a smaller forehead patch than in the previous year, while those whose broods had been reduced returned with a larger patch. Furthermore, young in enlarged broods have reduced growth rates⁹; and when sons which had been reared in these broods returned as adults, they had smaller forehead patches than those from reduced clutches.

What are the consequences of these manipulations for reproductive success in subsequent years? There was no evidence of changes in survival of males, nor in the timing of breeding, clutch size or fledgling number. This is surprising because one would expect there to be trade-offs between current reproduction and future fitness. For example, experiments with swallows, *Hirundo rustica*, have shown that males whose ornamental tail feathers had been elongated were slower to attract mates the following year, were less likely to have second clutches and produced fewer fledglings¹⁰.

However, other components of fitness in flycatchers may have been compromised by the manipulation. Male flycatchers can increase their fitness through polygamous pair-bonds. Gustafsson *et al.* showed that, in unmanipulated birds, a switch in male mating status from polygamy in one year to monogamy the next summer coincided with a decrease in forehead patch size between years. Conversely, males that went from having a single mate to several mates had larger forehead patches in the second year. The implication is that changes in body condition from one year to the next may have been signalled by forehead patch size, leading to changes in mating success of males.

The new study¹ shows that neither sex-

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REASONS

Status symbol: a collared flycatcher displays its prominent white patch.

Roger Wilmshurst

1. Gustafsson, L., Qvarnström, A. & Sheldon, B. C. *Nature* **375**, 311–313 (1995).
2. Darwin, C. R. *The Descent of Man, and Selection in Relation to Sex* (Murray, London, 1871).
3. Andersson, M. *Sexual Selection* (Princeton Univ. Press, 1994).
4. Johnstone, R. *Biol. Rev.* **70**, 1–65 (1995).
5. Williams, G. C. *Am. Nat.* **100**, 687–690 (1966).
6. Stearns, S. C. *The Evolution of Life Histories* (Oxford Univ. Press, 1992).
7. Clutton-Brock, T. H. & Parker, G. A. *Nature* **373**, 209–216 (1995).
8. Hansen, A. J. & Rohwer, S. *Anim. Behav.* **34**, 69–76 (1986).
9. Gustafsson, L. & Sutherland, W. J. *Nature* **335**, 813–817 (1988).
10. Møller, A. P. *Nature* **339**, 132–135 (1989).
11. Houston, A. I. & Davies, N. B. in *Behavioural Ecology: An Evolutionary Approach* (eds Sibly, R. M. & Smith, R. H.) 471–487 (Blackwell, Oxford, 1985).

ual selection nor life-history trade-offs should be considered in isolation. For male collared flycatchers, the costs of caring for larger broods may include reduced sexual competitiveness for themselves in future years, and for their sons once they reach maturity. This remains to be shown directly, but it is to be expected from the reduction in the patch size of both fathers and sons. Fathers should therefore adjust their parental care to balance a trade-off between their own sexual competitiveness in future years and that of their sons. In addition, females have been shown to respond to an increase in clutch size by a reduction in future fecundity¹⁰. Game-theory models predict that each parent should adjust its parental care according to care by the other¹¹, so life-history effects on females should have

repercussions for males.

It is intriguing that forehead patch size should serve as such a sensitive badge of condition in both generations. Because this trait is probably not costly in energetic terms, reliability of the signal must be enforced by social interactions. It would be interesting to know whether other sexual traits which are more energetically expensive, such as singing and courtship displays, are as reliable as forehead patch size as indicators of condition. The evidence from this study is that life-history trade-offs may leave animals with little room for dishonesty in sexual selection. □

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B CELLS

Avoiding autoreactivity

Ian C. M. MacLennan

GERMINAL centres arise when B cells activated by antigen undergo massive clonal expansion in the follicles of secondary lymphoid organs. The B cells activate a site-directed hypermutation mechanism which modifies the genes encoding their antigen-specific receptors¹. This somatic mutation can markedly increase the affinity of the germinal centre (GC) B cells for antigen, but it may reduce affinity or alter the specificity of the receptor so that it becomes autoreactive. Papers by Pulendran *et al.*² and Shokat and Goodnow³ (pages 331 and 334 of this issue) address the latter problem.

A rigorous selection process acts on B cells before they can leave germinal centres to become antibody-secreting cells or memory B cells: the proliferating GC B cells come out of cell cycle and move to a part of the germinal centre that contains a network of dendritic cells, which take up and hold unmodified antigen⁴. In this site the B cells soon die by apoptosis unless they receive positive antigen-specific selection signals⁵. From the available evidence it seems that selection requires the B cells to take up antigen from the follicular dendritic cells, process it and present it to local T cells (reviewed in ref. 6).

The two new papers^{2,3} show that a greatly increased proportion of GC B cells die by apoptosis upon injection of a large amount of soluble protein that is bound by their antigen-specific receptors. The injected soluble protein would be expected, by competitive inhibition, markedly to reduce the chance of GC B cells binding to the relatively small amount of antigen held on follicular dendritic cells. In some of the experiments, injected soluble protein bound to the B cells' receptor for

antigen but lacked peptides recognized by the GC T cells. In this situation the GC B cells would be expected to die because injected soluble protein would not enable these cells to elicit positive selection signals from GC T cells. There seems to be another cause of the greatly increased GC cell death seen by Pulendran *et al.* and Shokat and Goodnow, for the same phenomenon occurred when a large amount of soluble antigen, identical to that used to induce the GC response, was injected. This antigen, of course, would have been visible to GC T cells.

The mechanism responsible for this effect remains unresolved, but a number of possibilities should be investigated. Massive blockade of GC B cells' antigen receptors by itself might induce apoptosis; such an effect has been reported with cell lines from Burkitt lymphoma, a B-cell neoplasm of germinal centre origin⁷. It is also possible that antigen has to be taken up from follicular dendritic cells for the selection to proceed successfully. Treatment of isolated GC B cells with soluble antibody against their surface antigen receptors fails to prevent spontaneous apoptosis, but the antibody bound to erythrocytes inhibits apoptosis⁸. Isolated GC B cells are protected from apoptosis when they bind to follicular dendritic cells *in vitro*, both binding and protection being prevented by antibodies that block adhesion proteins carried on these cells⁸. Germinal centre cells take up, from follicular dendritic cells, antigen which they process and present to T cells⁹. Analysis of their ability to do the same with different concentrations and forms of soluble antigen is called for.

The induction of apoptosis in GC cells

with soluble antigen was still seen in transgenic mice whose B cells constitutively expressed Bcl-2 (ref. 2), a molecule that is absent in normal GC cells and that can inhibit apoptosis in certain cells¹⁰. This suggests that the effect of soluble antigen *in vivo* may be to induce apoptosis rather than prevent selection signals being received. On the other hand, T cell-derived signals that prevent apoptosis in GC B cells act independently of up-regulation of Bcl-2, although they do induce the expression of this molecule¹¹. Susceptibility of Burkitt lymphoma lines to induced apoptosis is only inefficiently inhibited by transfected *bcl-2* unless it is markedly overexpressed¹². These findings imply that susceptibility of GC B cells to enter apoptosis spontaneously is only partially explained by lack of Bcl-2 expression.

Can any physiological significance be attributed to the new finding^{2,3}? The phenomenon might cause the demise of GC B cells that acquire autoreactivity to proteins in the extracellular fluids through mutation of the genes encoding their antigen receptors. Serum immunoglobulin and derivatives of the third component of complement bind to antigen on follicular dendritic cells. Consequently, a GC B cell with acquired affinity for self-immunoglobulin might take up foreign antigen complexed with antibody from follicular dendritic cells. The B cell could then receive selection signals by processing and presenting peptides from the foreign antigen to local T cells.

Patients with rheumatoid arthritis develop antibodies that react with their own immunoglobulin. These can be high-affinity antibodies encoded by somatically mutated genes for the immunoglobulin-variable region¹³. In rheumatoid arthritis the mechanism described by Pulendran *et al.* and Shokat and Goodnow seems to have failed, but it might protect most of us from developing these autoantibodies. □

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- Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. *Nature* **354**, 389–392 (1991).
- Pulendran, B., Kannourakis, G., Nouri, S., Smith, K. G. C. & Nossal, G. J. V. *Nature* **375**, 331–334 (1995).
- Shokat, K. M. & Goodnow, C. C. *Nature* **375**, 334–338 (1995).
- Mandel, T. E., Phipps, T. E. R., Abbot, A. & Tew, J. *Immun. Rev.* **53**, 29–59 (1980).
- Liu, Y.-J. *et al.* *Nature* **342**, 929–931 (1989).
- MacLennan, I. C. M. *A. Rev. Immun.* **12**, 117–140 (1994).
- Gregory, C. D. *et al.* *Nature* **349**, 612–614 (1991).
- Koopman, G. *et al.* *J. Immun.* **152**, 3760–3767 (1994).
- Kosco, M. H., Szakal, A. K. & Tew, J. G. *J. Immun.* **140**, 354–360 (1988).
- Pezzella, P. *et al.* *Am. J. Path.* **137**, 225–232 (1990).
- Holder, M. J. *et al.* *Eur. J. Immun.* **23**, 2368–2371 (1993).
- Milner, A. E., Johnson, G. D. & Gregory, C. D. *Int. J. Cancer* **52**, 636–644 (1992).
- Deftos, M., Olee, T., Carson, D. A. & Chen, P. P. *J. clin. Invest.* **93**, 2525–2553 (1994).

Abandon all models

Leslie Sage

IN April 1920, a great astronomical debate was held between Harlow Shapley and Heber D. Curtis in the auditorium of the Museum of Natural History in Washington DC. That debate centred on the nature of the 'spiral nebulae', which we now know are other galaxies, but which many astronomers at that time (including Shapley) held to be similar to the gaseous nebulae surrounding massive young stars and now identified as regions of ionized hydrogen.

Partly to mark the 75th anniversary of the event, a debate on one of today's most contentious astronomical problems was held in the same auditorium on 22 April this year. Twenty-six years after the discovery of bursts of gamma-ray emission from space, the source of these very-high-energy photons is as mysterious as ever. Donald Lamb and Bohdan Paczyński, leading proponents of the two major prevailing views, put forward their opinions on the relevant issues.

Paczyński started the debate by stating that there are two camps, but that they are not the ones usually recognized. He said that there are those who acknowledge that they have no idea what is causing the bursts, and those who do not acknowledge that basic fact. All we really know about the gamma-ray bursters (GRBs) is that they are distributed more or less isotropically across the sky. There are only about 1,100 recorded bursts, making it difficult to determine whether the distribution is truly isotropic, and therein lies the source of the controversy.

Most objects associated with our Galaxy are not distributed isotropically, except for a restricted sample of nearby stars. There is an inherent anisotropy built into the Milky Way because of its disk-like structure, which is easily seen in the bright band of stars across the night sky. The only other objects in the Universe that are distributed in a relatively uniform fashion across the sky are very distant galaxies. (Although there seem to be structures in the distribution of galaxies, we will regard their distribution as isotropic for simplicity.) Even the globular clusters, which are distant compact groups of a million or more stars orbiting the centre of the Milky Way, are not isotropically distributed from our perspective in the outer part of the Milky Way. They are definitely clustered around the centre, in the direction of the constellation of Sagittarius.

This relative isotropy suggested to many people that the GRBs are either pretty local (originating within a few hundred parsecs of the Sun) or lie at distances comparable to the most distant galaxies. As instruments have improved, particu-

larly with the launching of the Compton Gamma-Ray Observatory by NASA in 1991, the so-called cosmological origin (perhaps associated with very distant galaxies) has come to be favoured, because the distribution has remained isotropic. But on the opposite side of the debate, the 'local' option, championed by Don Lamb, now favours sources distributed in an extended halo around the Milky Way, with an average distance of 100 kpc.

As Lamb pointed out, the current data are consistent with this interpretation. Paczyński countered that the critical test will arise when we have more data, which should allow us to see an anisotropy in the distribution arising from our position 8 kpc from the centre of the Milky Way. In particular, there should be an excess of sources in the direction of M31, the nearest giant spiral galaxy to us. M31 lies at a distance of only 600 kpc, so if we can detect GRBs with an average distance of 100 kpc around the Milky Way, we should see an excess of relatively faint sources in the direction of M31. Lamb admitted that if this excess is not seen, and the distribution of sources continues to be isotropic around us, then he will have to abandon his position.

Paczyński suggested that the best way to make progress towards understanding the GRBs is to abandon all models. The astronomical literature is rife with proposed explanations, which range from colliding comets to merging neutron stars. More speculative sources include the exhaust from warp-drive engines. Paczyński maintained, however, that preconceived notions of what might be causing the bursts are biasing the debate about their distance scale. He recommended that we discard all current models until we have more information.

Despite the entertaining points made by both Lamb and Paczyński during the debate, I doubt that anyone entering the auditorium with preconceived notions left with a different opinion. The data are only preliminary, and some will undoubtedly prove to be wrong or misleading.

Shapley, who is generally considered to have 'won' the debate seventy-five years ago, was proven wrong within five years by Edwin Hubble. The decision about who won this year's debate seems less clear, although the majority opinion is in favour of a cosmological origin of the GRBs. Will history repeat itself, quickly demonstrating that the GRBs arise from a local population of sources? One thing is certain — the Universe yet holds many surprises for us. □

Leslie Sage is an assistant editor of *Nature*.

Watch this space

THE human eye trembles all the time, scanning the focused image over its retina at 30–80 Hz. This scan is crucial to vision. If an optical target is vibrated in synchronism with the scan, its image can be stabilized on the retina. It promptly disappears from view.

In principle, therefore, an agile dancer might vanish from a watcher's gaze, simply by shimmying at his optical scanning frequency. Daedalus is now making this scheme more practical. His 'cloak of invisibility' is a bulky, all-enveloping garment made of a liquid-crystal display material. An invisible infrared beam is projected through the fabric; it bounces off the watcher's eyes and retrieves their scanning motion. This is used to vibrate, not the cloak or its wearer, but the pattern displayed on the cloak. As synchronization locks in, it vanishes from the watcher's gaze.

What will the watcher make of this sudden blank in his field of view? He will not even be aware of it. The visual system automatically fills in a retinally stabilized gap with some plausible interpolation of the surroundings, just as it fills in our blind spot. This is big enough to take the images of 150 full moons, but we never notice it. Similarly, anyone looking at the cloak of invisibility will unknowingly extend the background scene right across it, and see nothing at all.

The cloak of invisibility will appeal to policemen, private detectives, spies, criminals, extramarital adventurers and many others wishing to blush unseen. Its main snag is that it can work perfectly against one watcher only. Two, each with his own scanning frequency, cannot be simultaneously unsighted. Even so, they may both be fooled. The watcher 'in sync' will see nothing at all. The other will see the wearer vanishing and reappearing at the beat frequency between the cloak's vibration and that of his own eyes. This could be just as baffling.

Indeed, Daedalus is planning a simpler cloak of invisibility whose pattern just vibrates unsynchronized at a range of frequencies around 50 Hz, varying from one point to another. Any watcher will then see the wearer broken up by wandering regions of invisibility, each filled in with some mental construct of his own. The static disruption of an expected outline is baffling enough: the dazzle-patterns worn by the ships of World War I made them hard to see or identify even against a blank horizon. Disruptions drifting with time will be far worse, and will utterly confuse the witnesses. Even if they manage to interpret the apparition correctly, their claims will seem incredible in court.

David Jones

The selfish pursuit of sex

SIR — No satisfactory explanation for the origin and evolution of sex in eukaryotes has been proposed that is based solely on the selective advantage (to organisms) derived from genetic shuffling, a primary consequence of meiosis¹. Several theories do see genetic shuffling as a byproduct of some other selective advantage, and one, 'the molecular symbiont' hypothesis, would have it as a primary advantage, but an advantage accruing to genetic parasites, not organisms¹. This idea, first proposed by Hickey² and by Rose³, has the weakness that no gene that is an integral

found in archaeobacterial DNA polymerase genes, indicates that HO endonuclease probably arose from a homing protein-intron at roughly the same time that *Saccharomyces* diverged from *Candida* (see figure), about 135 million years ago⁷. The alternative, that protein-introns are degenerate derivatives of HO endonuclease⁵, is a far less parsimonious interpretation of the phylogeny.

Although there are cases of selfish elements directing the fusion of mitochondria to promote their own spread in eukaryotes⁸, the relationship between HO endonuclease and protein-introns is the first strong evidence for a mobile element actually becoming integrated into the sexual cycle of the nucleus. To understand the significance of this relationship, one must appreciate the effect that frequent mating-type switching could have on the spread of a transposon. In a heterothallic population under certain conditions, such as a discontinuous or patchy population distribution with little mixing, subpopulations of essentially clonal lineages of one mating-type may arise. Haploid individuals from a population bearing an element that increases the frequency of mating-type switching would more often find compatible mates under these conditions: within their original clonal lineage they

mating-type switching was to favour diploidy, a measure that improved the organism's ability to repair damaged DNA⁶. We suggest, however, that by assuming this role in mating-type switching, the mobile protein-intron may have improved its own chances of propagation. If this proposal is correct, the establishment of HO in the ancestral *Saccharomyces* population was not primarily the consequence of organism-level selection for more frequent mating-type switching⁶, but rather may have been the result of a selfish transposon which had cleverly exploited a pre-existing mating-type switching process to speed its own spread.

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1. Hickey, D. A. *J. Hered.* **84**, 410–414 (1993).
2. Hickey, D. A. *Genetics* **101**, 519–531 (1982).
3. Rose, M. R. *J. theor. Biol.* **101**, 137–147 (1983).
4. Bell, G. J. *Hered.* **84**, 351–359 (1993).
5. Gimble, F. S. & Thorne, J. *Nature* **357**, 301–306 (1992).
6. Herskowitz, I. *Microbiol. Rev.* **52**, 536–553 (1988).
7. Berbee, M. L. & Taylor, J. W. *Can. J. Bot.* **71**, 1114–1127 (1993).
8. Hurst, L. D. *Nature* **354**, 23–24 (1991).

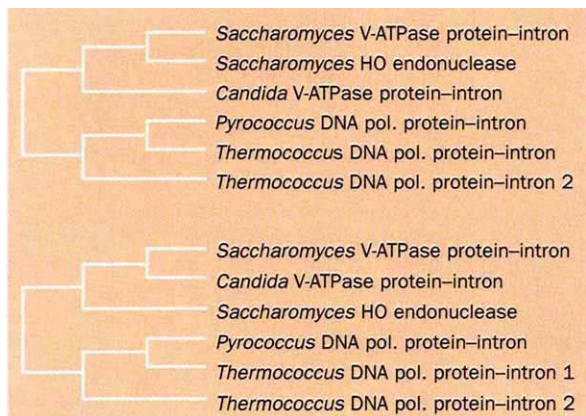


FIG. 1 Two possible phylogenetic relationships between HO endonuclease, fungal V-ATPase protein introns and archaeobacterial DNA polymerase protein-introns. The upper tree was obtained by parsimony analysis (using PAUP 3.1 program), whereas the lower tree was generated by neighbour-joining of a protein-distance matrix (using PHYLIP 3.5 programs) and protein maximum likelihood (using MOLPHY 2.2 programs), all inferred from 57 clearly alignable amino-acid positions. The exact topology of the fungal sequences cannot be discerned, but all methods confirm with strong statistical support that HO and the two ATPase protein-introns are most closely related and diverged at about the same time.

part of the eukaryotic sexual process has been shown to have been derived from a selfish mobile element^{1,4}. In fact, a good example has been in the literature for some time, but its significance has never been recognized.

Recently, a new class of selfish mobile element has been discovered, the protein-intron (intein). These elements are endonucleases that are also known to 'home', a particular type of mobility that uses double-stranded-break repair to invade uninfected alleles⁵. Interestingly, these mobile elements are also related to the endonuclease encoded by the homothallic switching (*HO*) gene in the yeast *Saccharomyces cerevisiae*, the factor responsible for initiating a mating-type switching cascade in this organism⁶. Closer examination reveals that HO is most closely related to the V-type ATPase protein-introns found in both *Saccharomyces* and the related *Candida tropicalis*. Phylogenetic analysis of these sequences, together with their next closest relatives, protein-introns

would be able to mate with close relatives lacking the element, and when introduced into a new subpopulation they will be able to outcross regardless of the resident mating-type. The immediate increase in accessible mates for this strain could thus facilitate the exposure of uninfected genomes to the element and favour its rapid spread throughout the population.

The mating-type switching system in contemporary *Saccharomyces* is a complex, highly regulated pathway in which HO has a single, central role. When HO arose, it may have become integrated into an already existing switching system that was less efficient, perhaps a passive system that relied only on gene conversion. The introduction of an endonuclease that cut the expressed mating-type locus in addition to its homing site could significantly increase the frequency of mating-type switching by forcing a gene conversion event to take place to repair the lesion.

It has been suggested that the primary selective advantage for the development of

Neanderthal computer skulls

SIR — Fossil specimens can be restored, measured and replicated without physical contact using a combination of computerized tomography (CT), computer-assisted reconstruction and rapid prototyping. We have used this procedure to reconstruct and reinterpret fragments of a Neanderthal child's skull from Devil's Tower in Gibraltar (Fig. 1a,b). We have developed an interactive software package to generate accurate object surfaces from serial CT data which permits three-dimensional morphometry and computer-assisted reconstruction of fossils. Rapid prototyping of computer-generated reconstructions was achieved using stereolithography (layer-by-layer production of successive thin profiles by laser-induced polymerization of a photo-sensitive liquid).

The Devil's Tower specimen is represented by five individual fragments: incomplete mandible, right maxilla, right temporal, fused frontal bones and left parietal. Of these original fragments, only the last two articulate directly. All fragments were originally attributed to a single individual¹ with an estimated age at death of 3–4 years^{2,3}; but it has also been suggested that the fragments came from two separate individuals of different ages⁴.

Using a graphics workstation equipped with a stereo monitor (Onyx, Silicon Graphics; Mountain View, California), three-dimensional skull reconstruction was achieved in four stages (Fig. 1a). (1) The

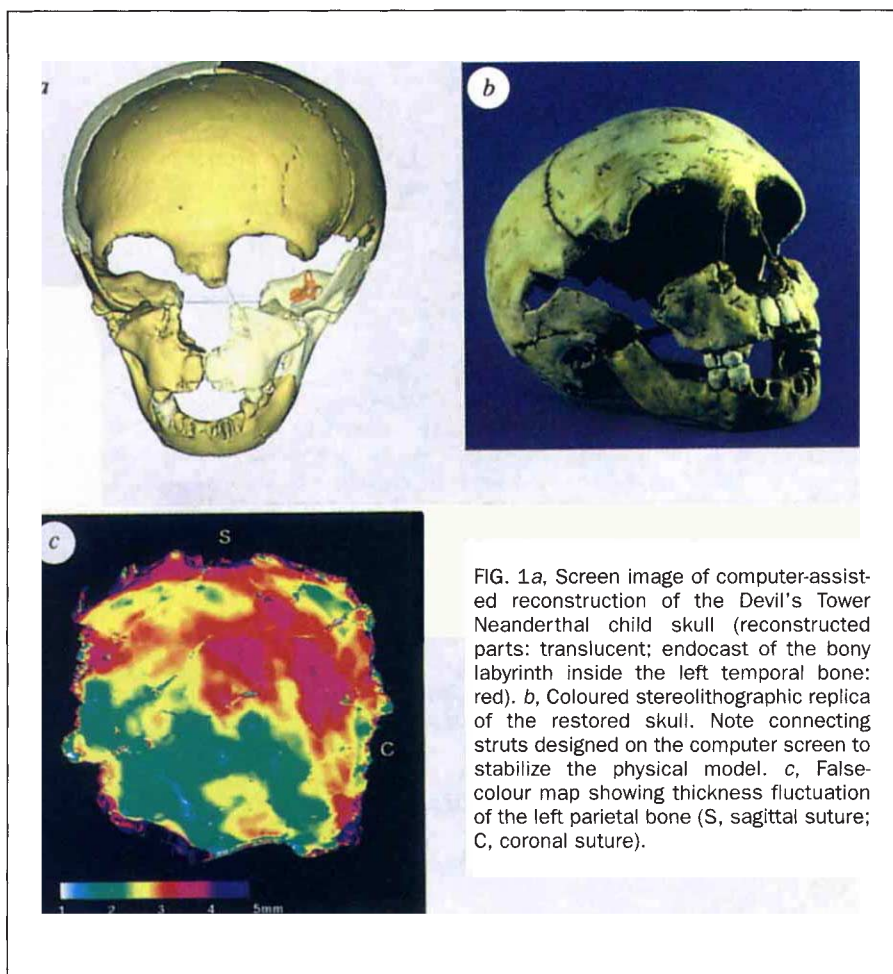


FIG. 1a, Screen image of computer-assisted reconstruction of the Devil's Tower Neanderthal child skull (reconstructed parts: translucent; endocast of the bony labyrinth inside the left temporal bone: red). b, Coloured stereolithographic replica of the restored skull. Note connecting struts designed on the computer screen to stabilize the physical model. c, False-colour map showing thickness fluctuation of the left parietal bone (S, sagittal suture; C, coronal suture).

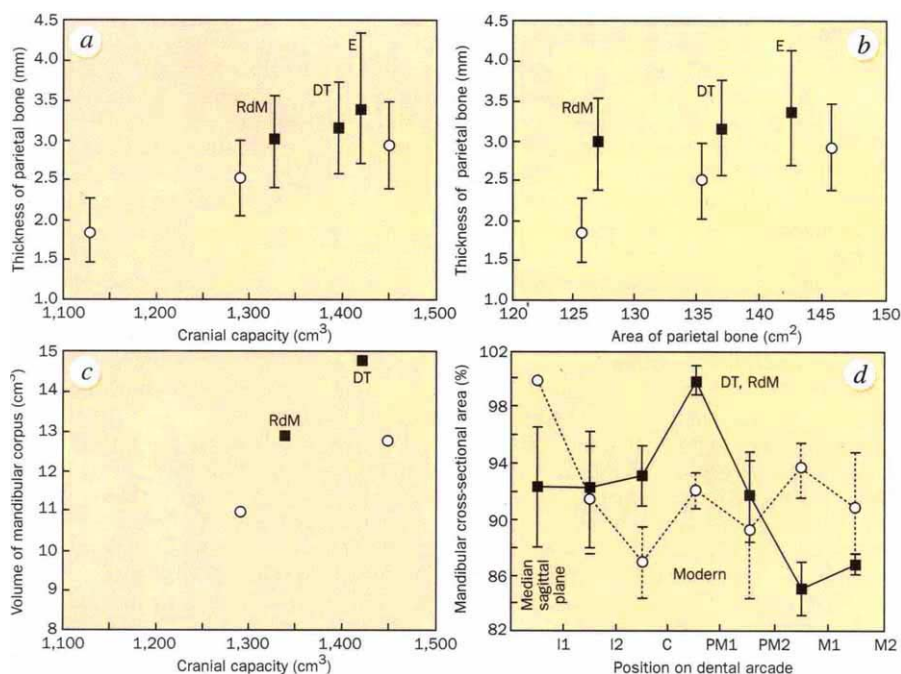


FIG. 2 Variation in cranial dimensions of Neanderthal children (DT, Devil's Tower; E, Engis 2; RdM, Roc de Marsal) and modern children of comparable dental age (3 individuals). a, b, Thickness of parietal bone versus cranial capacity and versus parietal surface area (squares, Neanderthal skulls; circles, modern skulls; bars indicate 50th±25 percentiles). c, Volume of mandibular corpus versus cranial capacity. d, Profile of the cross-sectional area of the mandibular corpus (pooled data, taking maximum values as 100% for each taxon; I, C, PM, M: positions of incisors, canine, premolars and molars, respectively).

missing left ascending ramus of the mandible and the right cheek teeth were restored using mirror images from the opposite sides. Following combination of the original right maxillary fragment with its mirror image, occlusion between the lower and upper cheek teeth could be established on the computer screen and checked with stereolithographic models. (2) The bony labyrinth inside the right temporal bone was visualized and used as an anatomical compass to orient the surrounding bone along the sagittal plane of the skull, defined by an angle of 45° relative to the planes of the superior and posterior semicircular canals^{5,6}. The oriented temporal bone and its mirror copy were then placed on the mandibular condyles. (3) The original left parietal and its mirror image were articulated with the fused frontal bones and fitted to the temporal bones already positioned in stage 2. (4) A small part of the orbital rim is present on the original maxillary fragment, so the height of the orbit and simultaneously the position of the jaws could be adjusted.

The very close fit between the independently reconstructed upper and lower portions of the skull (Fig. 1a) strongly indicates that the fragments indeed belong to a single skull, despite an apparent disparity between age estimations for dental eruption and development of the ear region based on modern human standards⁴. Our cohesive reconstruction indicates instead that dental eruption in Neanderthals was advanced in comparison with modern human standards, as suggested elsewhere².

Stereolithographic replicas of the skull (Fig. 1b) built on a SLA250 (3DSystems; Valencia, California) device using our software are accurate to ±0.7% of linear dimensions of the originals, matching the reliability of conventional casts. Indeed, stereolithography is superior to conventional casting because of its non-invasiveness and accuracy of replication of cavities and fragile features.

Computerized reconstruction also permits extensive morphometric analysis in one, two and three dimensions. We analysed three modern skulls and three Neanderthal child skulls, Devil's Tower (DT), Engis 2 (E) and Roc de Marsal (RdM), all of comparable dental age. To assess cranial capacity of the fragmentary fossil skulls, we completed missing parts of the endocranial cavities by 'morphing' data for existing parts. Subject to the

- Garrod, D.A.E. et al. *J. R. anthrop. Inst.* **58**, 33–113 (1928).
- Dean, M.C., Stringer, C.B. & Bromage, T.G. *Am. J. phys. Anthropol.* **70**, 301–309 (1986).
- Stringer, C.B., Dean, M.C. & Martin, R.D. in *Primate Life History and Evolution* (ed. DeRousseau, C.J.) 115–152 (Wiley-Liss, New York, 1990).
- Tillier, A.-M. Z. *Morph. Anthropol.* **73**, 125–148 (1982).
- Delattre, A. & Fenart, R. *L'homnisation du Crâne Étudiée par la Méthode Vestibulaire* (CNRS, Paris, 1960).
- Spoor, F., Wood, B. & Zonneveld, F. *Nature* **369**, 845–848 (1994).

accuracy of such morphing, fairly precise endocranial volumes could be determined: 1,400 cm³ for DT, 1,440 for E and 1,325 for RdM.

It is well known that Neanderthals have more robust cranial bones than modern humans, but it has been difficult to quantify this difference due to local fluctuation in bone thickness and variation in individual size. Computer-generated maps of the parietal bone (Fig. 1c) confirmed this fluctuation in thickness, but our capacity to sample at numerous points clearly revealed that average thickness of the parietal bone is elevated in Neanderthals relative both to parietal surface area and to cranial capacity (Fig. 2a,b). Similarly, Neanderthal mandibles are more robust in relation to the size of the braincase, and cross-sectional areas sampled along the dental arcade show distinctive profiles in Neanderthal and modern children (Fig. 2c).

In sum, using computer-assisted methods, marked quantitative differences in skull morphology can be demonstrated between modern humans and Neanderthals at an age of only 3–4 years. The existence of these clear quantitative differences at such an early age considerably strengthens the interpretation that Neanderthals and modern humans are separate species.

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Discontinuance of SMOW and PDB

SIR — Confusion exists in the reporting of stable hydrogen, carbon and oxygen isotope results, because the supply of the PDB (Peedee belemnite) standard has been exhausted and because SMOW (standard mean ocean water) does not have a unique definition. Because laboratories do not use the same reference material to establish their isotope-ratio scales, different laboratories report widely varying values for the same material.

To eliminate confusion in the reporting of light stable isotope ratios, the Commission on Atomic Weights and Isotopic Abundances of the International Union of Pure and Applied Chemistry has recommended^{1,2} that the use of SMOW and PDB be discontinued and that isotope abundances of hydrogen-, carbon- and oxygen-bearing materials be reported relative to the reference water VSMOW (Vienna standard mean ocean water) and to VPDB (Vienna Peedee belemnite), defined by adopting a $\delta^{13}\text{C}$ value of +1.95‰ and a $\delta^{18}\text{O}$ value of -2.20‰ for NBS 19 carbonate relative to

VPDB. The isotope abundance scales should be normalized so that SLAP (standard light Antarctic precipitation) has a $\delta^2\text{H}$ and $\delta^{18}\text{O}$ of -428 and -55.5‰, respectively, relative to VSMOW. If the isotope abundance measurement of a sample depends on an isotopic fractionation factor, this should be reported.

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1. IUPAC *Pure appl. Chem.* **66**, 2423–2444 (1994).

2. Coplen, T.B. *Pure appl. Chem.* **66**, 273–276 (1994).

Apolipoprotein E and Alzheimer's

SIR — The report by Games and colleagues¹ of the first successful generation of transgenic mice overexpressing pathological human β -amyloid precursor protein (APP) which also exhibit some of the pathological features of Alzheimer's disease is undoubtedly a milestone. Nevertheless, it may not yet be possible to conclude, as did Duff and Hardy in their News and Views article², that this result settles the argument for the primacy of amyloid deposition in Alzheimer's disease neuropathology as opposed to other factors such as apolipoprotein E4 (apo E4)³.

Although cysteine-containing apo E3 is by far the most frequently occurring isoform in humans, apo E4 is considered the ancestral form⁴. In most species, including mouse, apo E lacks cysteine and is E4-like, with arginine occupying the equivalent of amino-acid positions 112 and 158 (ref. 5). The findings by Games and colleagues cannot, therefore, exclude a role for apo E4 in the pathogenesis of Alzheimer's disease, as the mice in their study are by one definition homozygous for apo E4.

Indeed, their results could be taken further to implicate a role for apo E4 in the disease process. To exclude such a role for E4, the analyses should be repeated in the progeny from matings of their mice with those lacking apo E. It is interesting that transgenic mice lacking apo E and people with apo E deficiency appear neurologically normal^{6,7}.

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1. Games, D. *et al.* *Nature* **373**, 523–527 (1995).

2. Duff, K. & Hardy, J. *Nature* **373**, 476–477 (1995).

3. Hyman, B. T. *et al.* *J. Neurochem. exp. Neurol.* **53**, 427–428 (1994).

4. Weisgraber, K. H. *Adv. Protein Chem.* **45**, 249–302 (1994).

5. Chan, L. & Li, W.-H. *Curr. Opin. Lipidol.* **2**, 96–103 (1991).

6. Popko, B. *et al.* *J. Neurochem.* **60**, 1155–1158 (1993).

7. Lohse, P. *et al.* *J. Lipid Res.* **33**, 1583–1590 (1992).

Hypermutation in T cells questioned

SIR — Zheng *et al.*¹ investigated the occurrence of point mutations in rearranged T-cell antigen receptor genes in different compartments of the spleen of immunized mice. Because of the antigen used, the immune response is clonally restricted, mostly to V β 3 and V α 11 genes. The authors have amplified a subset of rearranged T-cell receptor genes from pools of 20–40 cells from the periaarteriolar T-cell sheath (PALS) and germinal centres (GCs), then subcloned and sequenced V(D)J gene segments.

The authors found that the error frequency of 14.2×10^{-4} per base pair for V α 11 segments recovered from GCs was much higher than for those recovered from the PALS (2.1×10^{-4}) or a T-cell line (0.7×10^{-4}). We would like to point out that even these large differences are not necessarily significant in replicating systems, which are intrinsically subject to large fluctuations. This is the fluctuation effect that Luria and Delbrück² used more than 50 years ago to prove that streptomycin-resistant bacteria were due to pre-existing mutations in the culture rather than to variants induced by the drug. Unfortunately, Zheng *et al.*¹ provide insufficient data for a proper quantitative analysis, and it is unlikely that the experiments were designed with that in mind.

The authors clearly believe that the mutations pre-existed PCR amplification. They say that a bias for mutations in non-productive alleles is indicative of counter-selection against mutants and thus inconsistent with “abiotic” processes. Again, the data provided do not allow one to decide whether there is selection against mutations, or no selection and merely fluctuation.

The successive accumulation of point mutations was first recognized by Weigert *et al.*³ to be the hallmark of affinity maturation plus hypermutation at immunoglobulin loci. Only in one clonally related family do Zheng *et al.*¹ find a successive accumulation of point mutations. Most unique mutations are found in this family. It is only a single clone, but we find this result to be much more suggestive than the claim of a quantitative difference between the GC and the PALS.

There is one problem. How do we know that the mutations arose in T cells? An equally tenable hypothesis would be that some GC B cells rearrange and hypermutate T-cell receptor genes. The authors cannot invoke the widely accept-

1. Zheng, B., Xue, W. & Kelsøe, G. *Nature* **372**, 556–559 (1994).

2. Luria, S. & Delbrück, M. *Genetics* **28**, 491–511 (1943).

3. Weigert, M., Cesari, I.M., Yonkovich, S.J. & Cohn, M. *Nature* **228**, 1045–1047 (1970).

ed evidence that T-cell receptor rearrangement is exceedingly rare in B cells and at the same time reject the even more widely accepted evidence that T cells do not hypermutate their T-cell receptor loci.

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KELSOE *ET AL.* REPLY — Bacht and Wabl are mistaken in stating that we presented insufficient data for a proper analysis of our results. Mutations in V α genes arise *in vivo* as a consequence of reaction in the germinal centre (GC) or *in vitro* during the PCR. Both processes are subject to Luria–Delbrück fluctuations and, in fact, the mutations observed in GCs fit a Luria–Delbrück distribution. However, the *in vivo* process is resistant to quantification since many factors are unknown (for example, replicative rates, independence of samples, effects of selection). In contrast, mutations introduced by the PCR can be rigorously analysed. Therefore, the null hypothesis that mutations in all samples were introduced *in vitro* can be tested.

We used a Monte Carlo algorithm to estimate the spectrum of PCR errors, including ‘jackpot effects’ due to early introduction of misincorporations, as follows. At each round of a simulated PCR, templates replicate with probability ϵ (0.8). If successful, each daughter independently incorporates a number of new mutations with parameter μ given by the product of the error rate of the *Pfu* polymerase and the length of the template

sequence¹; this is repeated for 80 cycles. This simulation was repeated 10⁴ times to estimate the median, mean, 99% and 99.9% quantiles for frequencies of V α 11/J α 11 rearrangements containing 1, 2 or ≥ 3 PCR errors. Predicted frequencies did not differ significantly from those observed in the B10 cell line (1 mutation observed versus 1.7 expected) and in periaerolar T-cell sheath (PALS) cells (7 observed versus 4.2 expected). In contrast, V α 11 mutations in GC cells were significantly in excess of expected PCR mistakes; for example, the number of V α exons with three mutations was about 3,500-fold above that expected. By chance, < 0.01% of sampled populations would contain triply mutated sequences at frequencies $\geq 1/4,500$; the frequency we observed for triple mutants in GCs was approximately 1/57.

Alternatively, problems of fluctuation may be avoided simply by scoring repeated mutations as single events. This analysis shows mutations in GCs to be significantly greater ($P = 0.03$; Fisher’s exact test) than that found in the PALS.

Bacht and Wabl correctly note that mutated V α rearrangements might originate in B lymphocytes. However, our present studies of single T cells dissected from GCs demonstrate V α 11 mutants in about 10% of productive rearrangements.

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velocities have an antisymmetrical behaviour relative to the centre of the disk, whereas the gravitational redshifts have a symmetrical one. The low-velocity lines should also be affected by a gravitational redshift of 4.0 km s⁻¹ as they are produced at the inner edge of the disk ($r = 0.13$ pc).

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Collision age

SIR — Beck *et al.*¹ have reported new biogeographical data, concluding that the collision of the Indian subcontinent with Asia was older than previously claimed. They suggested that the collision occurred between 66 and 55.5 Myr (million years) ago. However, palaeontological data constrain the latest possible date even more accurately. From the study of continental faunas and floras, it has been known for several years that terrestrial continuity between India and mainland Asia was already established by the time of the K/T boundary, 65 Myr ago, or probably slightly earlier^{2,3}. More recent data again support this view^{4,5}.

Nevertheless, an Eocene collision, which was accepted for a long time, appears still to be admitted by various workers. The biostratigraphical result of Beck *et al.* is an additional argument which demonstrates that such an age can no longer be accepted.

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1. Beck, R. A. *et al.* *Nature* **373**, 55–58 (1995).
2. Sahni, A. *et al.* *Bull. Soc. géol. Fr.* **14**, 1093–1099 (1982).
3. Hartenberger, J.-L. *Bull. Soc. Palaeont. Ital.* **21**, 283–288 (1982).
4. Prasad, G. V. R. *et al.* *J. Vertebr. Paleont.* **14**, 260–277 (1994).
5. Prasad, G. V. R. & Rage, J.-C. *Cretaceous. Res.* **16**, 95–107 (1995).

Redshift and black-hole mass

SIR — The very interesting observations by M. Myoshi *et al.* (*Nature* **373**, 127–129; 1995) of water-vapour maser emission in the NGC 4258 disk offer an additional direct test for their model of a high-rotation velocity disk and allow an independent determination of the central black-hole mass. Indeed, a detectable amount of gravitational redshift should affect the observed line wavelengths.

As the distances of the maser emission regions from the centre of the black hole r are much larger than the black-hole Schwarzschild radius $r_s = 2GM/c^2 = 3.5 \times 10^{-6}$ pc, the gravitational redshift is simply given by (assuming a spherical symmetry):

$$z(r) = \frac{r_s}{2r} = \frac{v_{\text{circ}}^2}{c^2}$$

where G is the gravitational constant, M the mass of the central object, v_{circ} the velocity of a particle in keplerian motion

around it at radius r and c the velocity of light. The gravitational redshift expected for a central mass of $3.6 \times 10^7 M_\odot$ at the inner edge of the disk is $cz(r = 0.13 \text{ pc}) = 4.0 \text{ km s}^{-1}$, whereas the one at the outer edge is $cz(r = 0.26 \text{ pc}) = 2.0 \text{ km s}^{-1}$, more than 10 times their velocity resolution, or about 4 and 2 times their quoted errors on the velocity determination for the high-velocity lines.

The high-rotation velocity lines offer an easy and safe way to test the disk model and to determine the black-hole mass. The reason is that they apparently arise very close to a line perpendicular to the line-of-sight. As a consequence, their velocities should systematically deviate from the values expected from keplerian motion, with residuals that are inversely proportional to the distances of the maser emission regions from the centre of the black hole. Further, the keplerian motion

The exploration instinct

Howard E. McCurdy

Pale Blue Dot: A Vision of the Human Future in Space. By Carl Sagan. *Random/Headline*: 1994/1995. Pp. 429. \$35, £25.

In *Pale Blue Dot*, Carl Sagan continues on the voyage of exploration that he began in his wildly popular book and television series *Cosmos*, a celebration of the human quest to understand the Universe. Here he stays somewhat closer to home, guiding readers through the wonders of our own Solar System. The journey comes none too soon. Initial probes, especially of the crater-pocked Mars, disappointed a generation of space enthusiasts raised on images popularized by such people as Percival Lowell and Edgar Rice Burroughs. Sagan's descriptions rekindle the sense of wonder necessary to maintain public interest in exploration.

The images Sagan presents are based extensively on findings from remotely controlled spacecraft that probed the Solar System during the past two decades. In February 1990, one of these spacecraft completed an assignment of little scientific but enormous philosophical value. The Voyager 1 spacecraft, well beyond and above the orbits of Neptune and Pluto, pointed its camera back along the path from which it had come and took a portrait of the Solar System.

There were the larger planets, bright dots stretched across the expanse of the void. Barely distinguishable against the glare of the Sun, a pale blue dot appeared. No civilizations, no great nations, no signs of world commerce could be seen — only a speck of blue against the cosmos. It would take a race of idiots, Sagan observes, to conclude that the small blue sphere called Earth by its inhabitants sat at the centre of creation. Using this image as a point of departure, he constructs the philosophy of space exploration that is the book's greatest contribution.

Astronomy is a humbling and character-building experience, he reminds us. For centuries humans took comfort in the belief that the Sun, Moon and stars rotated around the Earth and that God had created humans in His own image for a special purpose. Astronomy devastated these beliefs. The Earth is a pale blue dot orbiting an inconspicuous star on the outer reaches of one of at least 40 billion galaxies. There are certainly other solar systems with inhabitable planets and probably intelligent creatures on them. Humans were created through the mechanics of evolution and, according to Sagan, the best evidence currently available does not support the need for a grand designer.

"Human beings cannot live with such a

revelation," Sagan quotes the British journalist Bryan Appleyard as saying. Sagan seems to agree. The great demotions, as he calls them, have created a more mature view of nature, but they have also devastated the human spirit. Maturity is painful; it is easier to think like a child. The new view of the cosmos tends to breed a sense of hopelessness in human affairs.

Sagan believes that space exploration can revitalize the human spirit made sombre by astronomical discoveries. "Once we overcome our fear of being tiny, we find ourselves on the threshold of a vast and awesome Universe that utterly dwarfs — in time, in space, and in potential — the tidy anthropocentric proscription of our ancestors." Phenomenal discoveries will soon be made about our own Solar System, and new instruments will allow us to inspect objects orbiting nearby stars. Humans may move off the Earth and settle on other planets. The human race, as impious as Sagan admits this sounds, may join in the process previously reserved for gods by creating habitable planets out of lifeless spheres. All this, in Sagan's view, will excite epochs of discovery far more enriching than those possible under the doctrines of old.

People were meant to roam the cosmos, he further suggests. The need to explore is firmly set in the genetic make-up of our species, a product of natural selection. Nothing lasts forever, and only peoples who crave to move on protect their descendants from the inevitable catastrophes that follow. "For all its material advantages, the sedentary life has left us edgy, unfulfilled."

If Sagan is correct, then the pioneering philosophy he advances will be remembered thousands of years from now. As he freely admits, the obstacles are formidable. The cost of spaceflight is now far higher than space enthusiasts had ever predicted. A single shuttle flight to and from low-Earth orbit costs some \$400 million, hardly economical to the settlers who must travel the twenty-first-century equivalent of the Oregon Trail. Nearby spheres are far less hospitable than places on Earth as yet unsettled, but Sagan holds that the former can be transformed into habitable environments for human beings.

Sagan believes we have little choice. Humans take a foolish risk by binding themselves to Earth. In the long run, he argues, every galactic civilization is obliged to become space-faring for the most practi-

cal of reasons: staying alive. He is especially concerned about the inevitability of asteroid strikes, whose potential force far exceeds the destructive power of all the nuclear weapons left on Earth.

Sagan ultimately concludes that we will move into space not because of practical reasons such as mining the asteroids, but because it is in our nature to go. This philosophy draws him into a discussion of the saving power of frontiers, a position every bit as controversial today as were Copernican theories of the Universe four centuries ago. Modern historians are engaged in a major campaign to debunk the idea that ingenuity and democracy depend on the existence of frontiers.

A final word needs to be said about Sagan's writing style. Sagan stands in a line of popular science writers that includes figures such as Willy Ley and Wernher von Braun. But he is almost alone in his capacity for communicating the wonders of the cosmos. Ley's and von Braun's prophecies were lucidly written in their time, but they read like engineering manuals compared with Sagan's prose. If Sagan's vision succeeds, it will be due in no small part to his skill in communicating it. □

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Science Book Prize

JOHN Emsley has won this year's £10,000 Rhône-Poulenc Science Book Prize for *The Consumer's Good Chemical Guide: A Jargon-Free Guide to the Chemicals of Everyday Life* (W. H. Freeman/Spektrum, £18.99, \$24.95).

In a review in *Nature* **371**, 214 (1994), John Mann wrote that "this accessible and entertaining book deserves to be widely read by the general public and contains much information that will be of use to teachers of elementary chemistry courses. It may also persuade those journalists and environmentalists who persistently misrepresent chemicals to give chemistry the credit it deserves."

The winner of the junior prize is Jay Young's *The Most Amazing Pop-Up Science Book* (Watts). The prizes are awarded annually for the best science books published in the United Kingdom for a general readership.

Other shortlisted books in the adult category were *The Language Instinct* by Steven Pinker, *The Snows of Olympus* by Arthur C. Clarke, *The Book of Man* by Walter Bodmer and Robin McKie, *Journey to the Ants* by Bert Hölldobler and Edward O. Wilson and *The Covenant of the Wild* by Stephen Budiansky. □

Crash course

Richard Wilson

Hazards Due to Comets and Asteroids.
 Edited by Tom Gehrels. *University of Arizona Press*: 1995. Pp. 1,300. \$75.

WHOEVER heard of anyone being killed by the impact of an asteroid? Why then worry about such a silly, unlikely event? Why not worry instead about the real dangers that our civilization has brought us of radiation, asbestos and pesticides?

These were, until recently, people's responses when I talked to them about possible threats from asteroids and comets. The public largely ignored the evidence presented in *Science* in 1980 by L. W. Alvarez and colleagues that one or more comets or asteroids weighing 10^{15} – 10^{16} kg had struck Earth 66 million years ago, and may have been responsible for the extinction of the dinosaurs. A NASA workshop in 1992 on the detection of near-Earth objects fared no better in persuading people of the dangers, with the *Wall Street Journal* laughing at the idea in an article headed "Never Mind the Peace Dividend: The Killer Asteroids are Coming".

But public and official complacency and misdirection were modified when an article by C. R. Chapman and D. Morrison appeared last year in *Nature* (367, 33; 1994). In it, the authors described the average lifetime risk of being killed by an asteroid impact as 5×10^{-4} . This is more than 50 times the lifetime risk to which the US Environmental Protection Agency (EPA) pretends to regulate, and ten times the risk of being hit by a falling aircraft (averaged over the whole population of the United States). What is more, this arcane subject was made more real by the collision of comet Shoemaker-Levy with Jupiter in July 1994.

Our immediate response to the prospect of Earth being hit by such objects is fatalistic; we can do nothing about natural disasters. This fatalism, although commonly adopted by the general public and much of the environmental movement, is misplaced. We can often do more to avoid natural disasters or to mitigate their effects than is generally admitted.

So it is particularly useful to have this collection of articles on the detection and mitigation of hazardous asteroids and comets. The articles, which arose from four recent workshops having their roots in a meeting in 1981, fall into eight parts, the titles of which convey the range of the discussions: Small Bodies; Searches, Orbit Determination, and Prediction; Near-Earth-Object Populations and Impact

Flux; Physical Properties; Space Exploration; Effects of Near-Earth-Object Impact; Hazard Mitigation; and Considerations for Future Work.

The title of the first part belies its importance. 'Small bodies' means bodies less than 10 km in diameter, large enough to cause a lot of trouble. Objects reaching Earth's atmosphere can be divided into three classes: small ones with diameters less than a few tens of metres that burn up in the outer atmosphere and merely add to the average dust levels (although dust has an important influence on the world's climate, its effect is indirect and is at present uncertain); intermediate-sized ones of 50 metres in diameter that penetrate to the troposphere and release kinetic energy equivalent to that yielded by 20

arrives every few hundred years.

In describing environmental 'perturbations' caused by such impacts, the authors set the threshold for a global catastrophe as the collision of a 1.5-km asteroid with an energy equivalent of 300,000 megatons. This would directly kill a few million people, although more than a billion would be affected indirectly. Evaporated debris from fires and the initial explosion would cause loss of daylight for several months and global cooling on a massive scale. It was realized 20 years ago, as we contemplated all-out nuclear war, that these indirect effects can happen as soon as a threshold has been crossed. These large-object risks dominate the average risk from asteroid impact, so uncertainty in the average risk is dominated by uncertainty in the estimation of these indirect effects.

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The only serious proposal for hazard mitigation is to detect the orbit of the incoming asteroids and to send up an explosive device — nuclear of course — to 'nudge' the asteroid into a trajectory that does not intersect Earth's orbit. The detection involves an array of telescopes to give up-to-the-minute information about any asteroids on a collision course. It is probably this concept that raised the ridicule of the *Wall Street Journal*. To many people the whole idea seemed to be an excuse for keeping alive the Strategic Defense Initiative (SDI, better known as the 'Star Wars' project), and employing its advocates, long after it was shown not to work — an initiative that with the end of the Cold War is irrelevant anyway. But, unlike the SDI calculations, the risk estimates of asteroid impact are reliable and are widely accepted. They may well be the only useful outcome of the initiative.

It will be interesting to see how these estimates alter public perception. The asteroid risks are calculated more reliably than most of the much smaller risks that the EPA regulates. Will comparisons force the EPA to recognize (at last) that lifetime risks of 10^{-4} are common and that there is no point in regulating smaller ones? I fear, however, that bringing in a new, though larger, risk for comparison will do little to displace concern for the older smaller ones; it may instead merely make people more sensitized to all risks.

Some 20 years ago, I pointed out that people seem to be more worried about accidents with large consequences and low probabilities than the 'average' risk would seem to justify. There is a fascination with the occasional fire in a theatre that kills 100 people, even though overall far more are killed in small fires. The possibility of a large nuclear-power accident troubles people despite the fact that they

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Keeping an eye on the competition — Sean Connery and Karl Malden in the 1979 film *Meteor* (Paladium Pictures).

megatons of TNT (assuming an object velocity of 30 km per second); and those of 1.5 km and more that hit the Earth with an energy equivalent of over a million megatons. The last of these categories includes the asteroid that crashed into Earth 65 million years ago, excavating the Chicxulub crater of 200 km in diameter on Mexico's Yucatan peninsula. With an energy equivalent of nearly 100 million megatons, this impact is believed to have been responsible for the mass extinction at the Cretaceous-Tertiary boundary layer.

Remarkably, it is now possible to estimate the distribution of objects in near-Earth orbit to within a factor of three. For the small asteroids that burn up in the upper atmosphere we can do this by looking at the cratering on the Moon as well as that on some large asteroids, as revealed by the Galileo space probe. For the larger ones we can use the craters on Earth together with radiometric dating.

For many years we have been aware of the Tunguska asteroid that fell over Siberia in 1908. Although it fell in a remote area, scientists have often speculated on the million or so people who would have died if it had fallen over New York City. An asteroid of this size (about 50 metres in diameter with an energy equivalent of more than 15 megatons)

know that the chance of it happening is small. I suggested weighting the risk by the number of people involved. Under this criterion, the asteroid impact risk should dominate public concern more than all other risks so far calculated.

The "risk bill" that has just been passed by the US House of Representatives and that will shortly come before the Senate would force such comparisons. The bill is already being attacked by those who prefer the *status quo* and want to stop particular activities they hate even if the risks are minuscule. This volume should play its part in that debate. Whatever the result, it makes interesting reading and will stimulate further ideas about the Universe we live in, however temporarily. □

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Growth factors

Mark Nesbitt

The Emergence of Agriculture. By Bruce D. Smith. W. H. Freeman/Scientific American Library: 1995. Pp. 231. \$32.95, £19.95.

THE search for the origins of agriculture has fascinated archaeologists, geographers and biologists for the past 40 years. This is partly because farming had a critical role in creating the agricultural surpluses that underpinned the evolution of towns, cities and empires from the first villages, and partly because recent advances in understanding have been fuelled by the intriguing interplay of evidence from both archaeology and the natural sciences.

Although the concept of well defined areas of origin remains central to ideas about the beginnings of agriculture, a welcome feature of this book is that it also assesses other candidate regions still poorly known. The origins and spread of farming are set firmly in the archaeological context of the first farmers and their hunter-gatherer predecessors. This is an ambitious task, and it is hardly surprising that it is the first such survey aimed at a general audience to have appeared in a generation. A real strength of the book is that it successfully provides worldwide coverage, not just for plants but animals too. The regional case studies are supported by good maps and many well chosen photographs; this is a handsome and readable book.

Beginning with the earliest known and most fully studied region of agricultural origins, the Near East, Bruce Smith clearly outlines the evidence of the distributions of wild ancestors of domesticated

plants and animals. The simple principle — that a species will probably have been taken into domestication within the native area of its wild ancestor — has been refined by the use of biochemical markers, as elegantly demonstrated by recent work unravelling the history of maize in Central America. When biological information of this type can be combined with the study of excavated plant and animal remains from archaeological sites in the same area, the region, date and modes of domestication can be identified with some precision. As the author carefully points out, however, this ideal state is rare. In large parts of the world, few early sites have been excavated, and from these even fewer biological remains have been recovered. In sub-Saharan Africa and all of the tropics, we can only speculate about agricultural origins, drawing on modern botanical evidence of wild ancestors. Surprisingly, there is no mention of recent advances in the identification of vegetative plant remains that are likely to transform our understanding of the origin of root crops in the tropics.

A range of new techniques have transformed the study of ancient organic remains. Use of the scanning electron microscope is illustrated by looking at new evidence for indigenous domesticates in eastern North America, such as marsh elder, sunflower and goosefoot. Accelerator radiocarbon dating, which can reveal the age of individual seeds or bones, has dramatically re-dated many of the most famous Middle and South American plant remains, now found to be two or three thousand years younger than first thought. The use of these two techniques has stimulated a major re-evaluation of agricultural origins in eastern North America, establishing the region as an independent centre of domestication before the arrival of maize agriculture from the south.

Agricultural origins are particularly subject to archaeological nationalism — it seems as if everyone would like to claim the first farmers for their own country. Smith adopts a refreshingly cautious approach to archaeological evidence, and takes care to explain problems in dating and identifying early domesticates. But the cautious approach is dropped for archaeological evidence for plant domestication in the Near East. The focus on a

narrow region of the Jordan valley ignores increasing evidence of early Neolithic sites elsewhere in the fertile crescent. The choice of the Israeli site of Netiv Hagdud for a case study of a "typical" early agricultural site is unfortunate: analysis of the plant and bone remains strongly suggests that this was a purely hunter-gatherer village. Similarly, the author is uncritical in his approach to the origins of rice agriculture in China, for



Entitled *Wheat*, this 1967 painting by Thomas Hart Benton illustrates many of the ways in which domesticated plants respond to deliberate planting and harvesting by humans: the plants develop a uniform size and their seeds ripen uniformly in time, are retained until harvesting and become conveniently packaged in terminal clusters. The picture is reproduced from the cover of *The Emergence of Agriculture*.

which both the dating (here pushed back to 8,000 years ago) and the region are likely to be controversial until direct radiocarbon dating and adequate publication of plant remains from the Far East becomes the norm.

This book brilliantly and lucidly synthesizes a huge scattered literature on the where, when and how of agricultural origins. In an all too brief consideration of the why — the reasons for the beginning of farming — Smith points to the origin of many early farming societies in sedentary, hunter-gatherer villages. Although perhaps overplaying the ease of the transition to agriculture, this is a welcome statement that agriculture needs to be studied in the context of a continuum of relationships between plants and people. Anyone interested in exploring human interaction with the environment, or the application of interdisciplinary techniques to an important problem in human behaviour, will enjoy this book. □

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Knowing minds

Cecilia Heyes

The Thinking Ape: Evolutionary Origins of Intelligence. By Richard Byrne. Oxford University Press: 1995. Pp. 266. £30 (hbk), £13.50 (pbk).

Mindblindness: An Essay on Autism and Theory of Mind. By Simon Baron-Cohen. MIT Press: 1995. Pp. 171. \$28.50, £17.95.

EVOLUTIONARY psychology uses evolutionary theory to weave cognitive, developmental, comparative and neuropsychology into accounts of the origins and nature of human thinking and behaviour. The resulting fabrics are, as these books illustrate, colourful and intriguing, but they are seldom alike, and often a little too luxuriant for their purpose. Richard Byrne and Simon Baron-Cohen disagree on important issues but they share an eagerness to go beyond current evidence in proposing models of cognitive development that will stimulate research.

Byrne outlines a three-stage model for the evolution of 'the thinking ape' — that is, human intelligence. Drawing on cladistics analysis and the behaviour of extant primates, he infers that interesting things began to happen in the primate lineage about 40 million years ago with the appearance of cebus-human ancestors (the now extinct common ancestors of cebus monkeys and modern humans). Their forerunners, lemur-human ancestors, were no brighter than other mammals. But, under selection pressure from their socially complex environments, the simian primates, starting with the cebus-human ancestors and including all monkeys and apes, evolved larger brains allowing more rapid and efficient associative learning, 'primed' by the actions of members of the same species.

These advances enabled cebus-human and macaque-human ancestors, like contemporary platyrrhine and catarrhine monkeys, to deceive and cooperate with one another. But they could not understand *why* their actions had certain effects on objects and other animals. This kind of 'insight' emerged about 16 million years ago with the orangutan-human ancestors and is confined, among today's primates, to the great apes. It represents a remarkable change in cognitive ability, including the capacity to mindread or attribute mental states to others, and allows forward-planning, tool-making, imitation and teaching. In Byrne's view, this second watershed in the origin of human intelligence evolved under selection pressure from the physical environment, and was a necessary precursor to the final stage, the evolution of language in hominids.

Byrne has provided both a readable

review of primatology, suitable for undergraduates, and an original and interesting thesis about the evolution of intelligence that will appeal to general readers as well as researchers in the field. The core of his theory is a now familiar claim: that there is a sharp discontinuity, "a Rubicon of cognitive capacity", between monkeys and apes. This view was elucidated, for a similar audience, by Dorothy Cheney and Robert Seyfarth in *How Monkeys See the World* (University of Chicago Press, 1990). But Byrne puts a new spin on the idea, not only through cladistics analysis, but also by suggesting that monkeys show primed associative learning and insisting that apes have more all-purpose intelligence than monkeys; they are not merely superior social schemers.

With his emphasis on the general nature of intelligence, one might expect Byrne to rely on laboratory data. But, mysteriously, he dwells on field studies and gives experiments short shrift. This will make the text engaging for the general reader. But specialists may find it less persuasive. After reading the book, they may still wonder why, if apes are so much more intelligent than monkeys, there is no evidence that they have larger brains or commonly engage in more complex behaviour in the wild. Byrne addresses these questions, but they strain his primary evidence of the "Rubicon in cognitive capacity" — anecdotal reports of intentional tactical deception, teaching and tool-use in chimpanzees.

In a sense, Baron-Cohen takes up the story where Byrne leaves off. Byrne leads us to a point in evolutionary history where most humans (and other apes) are born with the potential for mindreading, and Baron-Cohen discusses the ontogeny of mindreading, the way in which evolutionary potential is realized in human development and what happens if the process is blocked. He suggests that the process normally has four steps, involving the activation of four cognitive mechanisms — the intentionality detector, the eye-direction detector, the shared-attention mechanism and the theory-of-mind mechanism — and claims that autism is a result of an arrest between the second and third steps. According to Baron-Cohen, autistic people suffer from 'mindblindness'; they are largely unaware that other people have minds.

The first two mechanisms work from birth. In effect, the intentionality detector distinguishes animate from inanimate objects and attributes goals or desires to the former, whereas the eye-direction detector is sensitive to gaze direction and understands the relation 'sees'. In normal children, but not autistic ones, the two detectors are joined about 9 months after birth by the shared-attention mechanism. (The role of experience in this transition and others assumed by the model is not

clear.) This mechanism allows the infant to appreciate that he or she and another individual are attending to the same object; it forms 'triadic representations' and is necessary for the development at about 18 months of the theory-of-mind mechanism. This final step, the crowning theory-of-mind mechanism, establishes triadic representations involving the whole range of mental-state relations, including 'pretends' and 'believes'.

Although replete with acronyms, Baron-Cohen's prose is lucid and endearingly deferential. Leda Cosmides and John Tooby refer in their foreword to "Baron-Cohen's trailblazing research", but his own comments imply that he has done little more than pin together some ideas from Nicholas Humphrey, David Premack, Alan Leslie and others. Despite its sabre-rattling tone, the foreword is much closer to the truth. The theory is new and, as Baron-Cohen points out repeatedly, is consistent with the data. But it seems that he could have generated any number of developmental schemes consistent with the same data, and it is not always clear why he chose this one.

For example, why are the eye-direction detector and the shared-attention mechanism taken to represent the relation 'sees' when infants' sensitivity to gaze direction could be more modestly attributed to an understanding of the relation 'looks'? And why is there just one, massive step between the shared-attention and theory-of-mind mechanisms? Such questions hover over the first half of the book, but when Baron-Cohen begins to discuss autism, they disappear. Using his careful and ingenious experiments with autistic children and referring to a wealth of literature on the disorder, he argues persuasively that these children's core impairment is indeed mindblindness.

Baron-Cohen thinks that mindreading exists only in humans, that it is modular and that it co-evolved with language. Byrne finds mindreading in other great apes, takes it to be a manifestation of general intelligence and claims that it was a necessary precursor to the evolution of language. I hope these two evolutionary psychologists also disagree about the ethical implications of mindreading. Byrne apparently supports the proposal laid out in *The Great Ape Project* (eds Paola Cavalieri and Peter Singer, St Martin's Press/Fourth Estate, 1993) that 'human' rights should be extended to all great apes largely on the strength of the ability of these animals to mindread. But I trust that neither he nor Baron-Cohen would wish to deprive autistic people — sufferers from mindblindness — of the same rights. □

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The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution

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The crystallographically determined structure of a soluble fragment from the major envelope protein of a flavivirus reveals an unusual architecture. The flat, elongated dimer extends in a direction that would be parallel to the viral membrane. Residues that influence binding of monoclonal antibodies lie on the outward-facing surface of the protein. The clustering of mutations that affect virulence in various flaviviruses indicates a possible receptor binding site and, together with other mutational and biochemical data, suggests a picture for the fusion-activating, conformational change triggered by low pH.

MEMBRANE glycoproteins of enveloped viruses have essential functions in the viral life cycle, including receptor binding and membrane fusion. The properties of these proteins are therefore important determinants of host range, tissue tropism, virulence and induction of protective immunity. A structural approach to molecular mechanisms has so far been achieved only for influenza virus, by X-ray crystallographic analyses of its two membrane proteins, haemagglutinin (HA)¹ and neuraminidase (NA)². Isolation and crystallization of a soluble form of the envelope glycoprotein from tick-borne encephalitis (TBE) virus³, a flavivirus, now permits a similar undertaking for a quite different group of viral pathogens.

Flaviviruses are positive-strand RNA viruses with an outer diameter of about 500 Å (ref. 4). They are among the smallest viruses that contain a lipid-bilayer membrane. Most are transmitted to their vertebrate host by infected mosquitoes or ticks. Several are important human pathogens, including yellow fever (YF), Japanese encephalitis (JE), TBE, and the dengue (Den) viruses. Mature virions contain three structural proteins⁵: a core nucleocapsid protein (C), a small transmembrane protein (M) and a major surface protein (E). E is believed to mediate receptor binding and membrane fusion, and it induces protective immunity⁶. The monomer contains about 500 amino-acid residues, with two transmembrane segments at its carboxy-terminal end⁵. E forms dimers on the surface of mature virions at physiological pH³; exposure to acidic pH (below 6.5) induces an irreversible conformational change that includes rearrangement to trimers⁷. This conformational change is believed to be critical for fusion of viral and endosomal membranes after uptake by receptor-mediated endocytosis.

Virus assembly occurs at the membrane of the endoplasmic reticulum (ER) and leads to formation of immature particles containing prM (the glycosylated precursor to M), C and E⁵. These particles pass from the ER to the Golgi, and prM is cleaved to M during exocytosis, probably by a furin-like protease. Immature virions do not exhibit low-pH-induced conformational changes or fusion activity^{8–10}. It therefore appears that one function of prM is to prevent irreversible inactivation during transport of the virus through the acidic compartments of the *trans*-Golgi network.

Treatment of TBE virions with trypsin releases a dimeric, soluble fragment of E (sE)³. This membrane-anchor free fragment includes residues 1–395, most of the ectodomain. We describe here the structure at 2.0 Å resolution. Its architecture is very

different from that of influenza HA, which has analogous functions. Rather than forming a spike-like projection, TBE-E extends in a direction parallel to the viral surface. Its three-domain structure includes an immunoglobulin-like module likely to be involved in receptor binding. The nature of the interdomain linkages suggests a model for the low-pH-triggered conformational change, which has characteristics very different from those seen in influenza HA.

Structure determination

The structure was determined by molecular averaging of the multiple-isomorphous-replacement (MIR) electron densities from two different crystal forms, one containing a dimer in the asymmetric unit, the other a monomer (see legend to Table 1 for details). The high-resolution data from crystal form II (Table 1) were used for crystallographic refinement. The packing about two distinct crystallographic dyads in crystal form II (*P*₆,22) is conserved in form I (*P*₆). There is an extended dimerization surface about only one of these two dyads. We have confirmed that this is indeed the molecular dyad by electron microscopy of the dimeric sE fragment bound to an antibody specific for its C-terminal domain (T. Kirchhausen and F.A.R., unpublished observations).

Molecular architecture

The structure of the sE dimer is depicted in Fig. 1. The overall dimensions are ~150 Å × 55 Å × 30 Å. The long direction is perpendicular to the molecular dyad. The 'side' view in Fig. 1*b* shows that one face of the dimer is gently curved, perhaps to conform to the surface of the viral membrane, a spherical bilayer, roughly 430 Å in diameter. The convex, external face bears the carbohydrate, the loops that vary in size and sequence among flaviviruses, and the principal antigenic sites (see below). The concave, internal face contains the fragment's C terminus, which therefore projects towards the membrane. The sE dimer is elongated parallel to the membrane, and it has a radial extension of only about 30 Å. It is likely that the TBE virus particle has icosahedral symmetry (S. Fuller, personal communication), and we believe that the rod-like E-protein dimers form a lattice-like network on the external surface.

The monomer

The polypeptide chain of the sE subunit folds into three distinct domains (Figs 1 and 2): a central β-barrel (domain I), an elon-

TABLE 1 Structure determination

Crystal form I											
Space group $P6_1$ Cell parameters: $a = b = 143.41$ Å, $c = 119.98$ Å											
Resolution bins (Å)	<7.1	5.8	5.0	4.5	4.1	3.8	3.6	3.4	3.2	3.0	Overall
R_{sym} (%) [*]	4.3	4.7	4.8	5.2	5.7	6.7	7.9	10.7	15.7	21.4	7.6
Coverage (%)	88.4	89.3	89.1	91.2	92.1	92.5	94.6	94.3	95.3	94.9	92.2
Mean redundancy†	2.9	2.9	2.9	2.8	2.9	2.9	2.8	2.9	2.9	2.7	2.8
$\langle I \rangle / \langle \sigma \rangle$ ‡	13.1	8.7	12.8	10.7	10.6	9.4	8.4	6.2	4.2	3.1	8.4
Mean figure of merit											
Centric reflections	0.50	0.75	0.73	0.70	0.60	0.53	0.52	0.50	0.42	0.29	0.6
Acentric reflections	0.43	0.55	0.46	0.40	0.35	0.35	0.33	0.32	0.30	0.24	0.3
Number of reflections	1,224	1,569	1,857	2,135	2,385	2,622	2,854	3,031	3,227	3,378	24,282
Heavy-atom derivatives	(CH ₃) ₃ Pb ⁺ · CH ₃ COO [−] 10 mM		K ₃ UO ₂ F ₅ 1 mM		HgZn(CN) ₄ 1 mM		K ₂ OsO ₄ 5 mM		EMTS‖ 50 mM		K ₂ OsO ₄ 1 mM
Phasing power§	0.76		0.63		0.56		0.75		0.50	0.38	
Coverage (%)	80		90		70		73		85	85	
Crystal form II											
Space group $P6_122$ Cell parameters: $a = b = 144.04$ Å, $c = 118.96$ Å											
Resolution bins (Å)	<5.8	4.2	3.4	3.0	2.7	2.5	2.3	2.1	2.0	1.9	Overall
R_{sym} (%) [*]	8.7	7.7	10.0	13.0	19.0	20.0	21.5	22.0	22.0	27.0	10.8
Completeness (%)	91.3	99.8	99.5	99.8	99.3	96.5	88.1	79.7	52.1	22.0	77.5
Mean redundancy†	6.3	7.3	7.5	7.4	6.5	4.8	3.3	2.3	1.6	1.2	4.8
$\langle I \rangle / \langle \sigma \rangle$ ‡	5.5	6.7	5.7	4.6	3.4	3.0	2.9	2.8	2.7	2.3	6.4
Mean figure of merit											
Centric reflections	0.73	0.63	0.50	0.38							0.56
Acentric reflections	0.52	0.39	0.33	0.27							0.38
Number of reflections	2,014	3,531	4,395	5,166	5,780	5,858	6,045	6,023	4,032	1,739	44,583
Heavy-atom derivatives	(CH ₃) ₃ Pb ⁺ · CH ₃ COO [−] 10 mM					EMTS‖ 50 mM					
Phasing power 10–3 Å res.§		0.76				0.50					
Coverage (%) 10–3 Å res.		80				85					
Refinement											
R -factor¶	21.0	14.8	16.1	20.6	24.6	25.3	26.7	27.8	29.3	30.1	21.4
Number of reflections	1,821	3,193	3,961	4,623	5,213	5,284	5,468	5,406	3,628	1,589	40,186
Free R -factor ^{¶¶}	22.0	19.2	18.9	26.7	28.2	29.8	29.7	30.2	33.3	33.2	25.1
Number of free reflections	193	338	434	543	567	574	577	617	404	150	4,397
Atom model:											
Total number of atoms (non hydrogen)	3,193					Number of water molecules					151
Number of amino-acid residues	395					r.m.s. deviation in bond lengths (Å)					0.01
Number of sugar residues	1					r.m.s. deviation in bond angles (degrees)					1.7

Fragment sE from the Neudörfel strain of TBE virus crystallizes in two different crystal forms. Crystal form I was obtained under conditions similar to those previously reported³. The crystals grow in 1.4 M KCl, 14% PEG 4000, TRIS–HCl buffer pH 8. The space group is $P6_1$, $a = b = 143.4$ Å, $c = 120$ Å. There is one sE dimer (90K) in the asymmetric unit, with a solvent content of about 70%. The diffraction from these crystals is anisotropic, and the limit is about 2.8 Å along c^* and about 3.0 Å in directions normal to c^* . Crystal form II is closely related to form I. The space group is also hexagonal, $P6_122$, $a = b = 144.0$ Å, $c = 119$ Å. The crystals grow in 2.5 M KCl, 30% PEG 3000, TRIS–HCl buffer pH 8. In this case, the molecular dyad is crystallographic, so that there is a single monomer (45K) in the asymmetric unit, with solvent content similar to form I. Form II crystals diffract to better than 1.9 Å along c^* , and to at least 2.1 Å in the a^*b^* plane. Diffraction data were collected from both crystal forms, as well as for five heavy-atom derivatives of form I and two derivatives of form II using the synchrotron light sources at CHESS (F1), SSRL (7.1) and NSLS (X25). One-degree oscillation frames were collected on Fuji image plates or on a MAR detector (Stanford) for crystal form I and all derivatives. High-resolution native data from crystal form II were collected using 0.5 degree and 0.75 degree oscillations. Data were collected at -10 °C; each complete set contained data from 10 to 30 crystals. The individual X-ray frames were processed using program DENZO (Z. Otwinowski). The CCP4 suite for protein crystallography was used for scaling and merging the data. We used a version of ROTAVATA that calculates (and applies) anisotropic temperature factors to individual X-ray frames (B. Temple, personal communication). An MIR electron density map was calculated for each of the two crystal forms using phases from the heavy-atom derivatives listed in the table, computed with program MLPHARE (Z. Otwinowski). The transformation relating the two independent molecules in crystal form I and the transformations relating molecules in the two crystal forms were derived initially from the positions of the heavy atoms common to both crystal forms (Pb and Hg) and then refined by maximization of the correlation between the superposed MIR electron densities (ED). The average ED map of the three independent molecules (two in crystal form I and one in form II) was used to determine the molecular envelope by visual inspection (using program ENVEDIT²³) at 3.8 Å resolution. A real space iterative averaging procedure²⁴ was then applied to refine and extend phases. After convergence at 3.8 Å, the resolution was extended to 3 Å by shells of 2 Miller indices each. MIR phases were used as a starting point (cycle 0) for each new shell of phase extension, augmenting the phases determined from averaging at the previous resolution. The subsequent cycles used the calculated phases from the reconstituted averaged maps, until convergence of the correlation coefficient between calculated and observed structure factors. The mean phase change between the initial MIR phases and the final phases was between 65 and 70 degrees. The resulting ED map was interpretable, and it was used to trace about 75% of the polypeptide chain on a mini-map. Program O²⁵ was used to build a model from the $C\alpha$ positions read from the mini-map, placing about 75% of the side chains and building polyaniline for the remainder of the polypeptide. Initial rigid body refinement with program XPLOR²⁶, dividing the molecule into two separate domains allowed the determination of more accurate transformations. The resulting averaged map permitted a number of significant adjustments to the model, including addition of most of the remaining side chains. Refinement was then done using XPLOR²⁶ and the high-resolution data from crystal form II. Alternate rounds of simulated annealing, energy minimization and manual rebuilding in simulated-annealing omit maps, yielded the current model. The R -factor is 21.4% (free R -factor²⁷ 25.1%) for all measured reflections with Bragg spacings between 12 Å and 1.9 Å. Bulk solvent corrections for calculated amplitudes were applied, using the XPLOR 'solmask' parameters $solden = 0.36$ and $solrad = 0.25$ (ref. 26).

^{*} $R_{\text{sym}} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$, where I_{hi} is the i th measurement of the intensity of reflection h , and $\langle I_h \rangle$ its mean value.

† Mean redundancy, Defined as the ratio of the total number of reflections measured to the number of unique reflections.

‡ $\langle I \rangle / \langle \sigma \rangle$, Ratio between the average value of the intensities of all reflections in a given shell to the average value of the standard deviations of their measurements.

§ Phasing power, $\langle f_{\text{H}} \rangle / \langle E \rangle$, the mean value of the heavy-atom structure factor amplitude divided by the mean lack-of-closure error.

‖ EMTS, Ethyl-mercuro-thio-salicylic acid.

¶ R -factor: $\sum |F_{\text{obs}} - F_c| / \sum F_{\text{obs}}$, where F_{obs} is the observed amplitude and F_c is the amplitude calculated from the model. The sums are carried out over all reflections used in the refinement.

¶¶ Free R -factor, R -factor calculated for reflections not included in the refinement (ref. 11). In this case, we have left out 10% of the reflections.

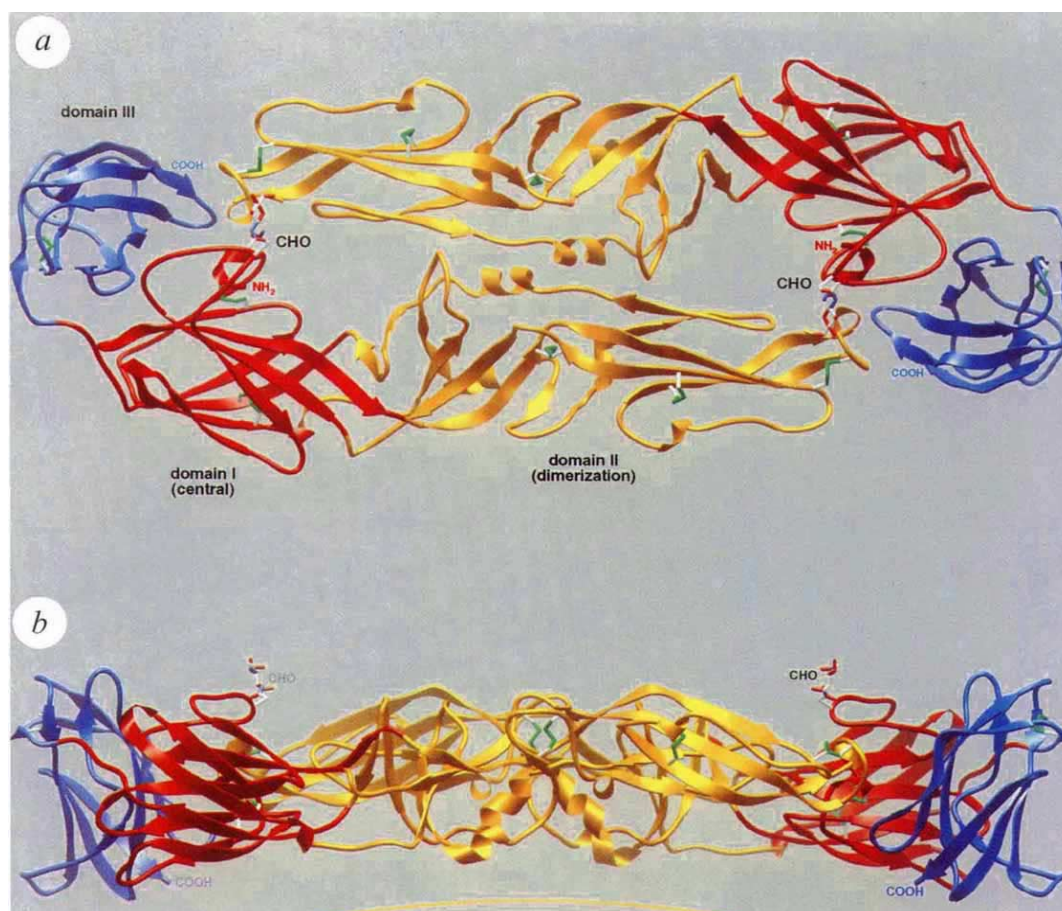


FIG. 1 Diagrams showing the folded structure of the TBE-sE polypeptide chain in the dimer. β -Strands are represented by ribbons with arrowheads; α helices, by coiled ribbons; connecting loops by thin tubes. The disulphide bridges are shown by ball-and-stick representations of the cystine residues. The single carbohydrate moiety, labelled 'CHO', is also drawn as ball-and-stick. The domains are coloured as follows: domain I, red; domain II, yellow; domain III, blue. *a*, The dimer viewed along the molecular dyad from a direction corresponding to the

outside of the virus particle. The total buried surface area per monomer is about $1,600 \text{ \AA}^2$ (about $700 \text{ \AA}^2$ across the 2-fold axis in the centre and $450 \text{ \AA}^2$ in each of the outer contacts on either side). The intrasubunit interface between domains I and III buries a surface of $650 \text{ \AA}^2$ on each domain. *b*, The dimer viewed from the 'side' (normal to the molecular dyad). The complete E protein would lie on the surface of the viral membrane, and a rough indication of the location and thickness of the lipid bilayer is shown by the curved lines. Drawn with RIBBONS²⁸.

gated dimerization region (domain II) and a C-terminal, immunoglobulin-like module (domain III). In all three domains, β -strand secondary structures predominate (Fig. 2*b*).

Domain I. The central domain I contains about 120 residues in three segments (residues 1–51, 137–189 and 285–302). The two loops between the segments form the dimerization domain II described below. The fold of the central domain is an eight-stranded up-and-down β -barrel (B_0 to I_0 in Fig. 2*b*, centre), with the addition of a short amino-terminal strand (A_0) parallel to strand C_0 at one edge. The axis of the barrel lies roughly parallel to the viral membrane. The two β -sheets of the domain face each other across a tightly packed hydrophobic interior. Sheet $A_0C_0D_0E_0F_0$ is on the external surface, with the unique glycosylation site on loop E_0F_0 ; sheet $G_0H_0I_0B_0$ faces the viral membrane. The barrel is open at one edge, where the N-terminal strand A_0 intervenes, and closed at the other, where two β -sheet hydrogen bonds join strands F_0 and G_0 . There are two disulphide bonds in this domain (Fig. 2).

Domain II. The large loops connecting strands D_0 and E_0 and strands H_0 and I_0 of domain I fold together to form a dimerization domain (residues 52–136 and 190–284). It is an extended, finger-like structure (Fig. 2*b*, top). Its base is an antiparallel β -sheet of five short strands (gfeah), with two α -helices packed against one surface. The elongated part of the domain is a narrow sandwich of a three-stranded β -sheet, containing residues from the D_0E_0 loop of domain I, and a β -hairpin, containing

residues from the H_0I_0 loop. The three-stranded sheet (bdc) is crosslinked by three disulphide bridges. The cd loop at the tip of the domain is contained within a sequence (residues 98–113) that is almost fully conserved in all flaviviruses (Fig. 2*a*). This sequence is quite hydrophobic and rich in glycine. It has been proposed that it is important for the fusogenic activity of the virus¹¹. The cd loop, which is packed against the other subunit of the dimer, has a tightly folded conformation, probably stabilized by the disulphide that links the end of strand b with the beginning of d (see Fig. 3).

At the junction of the central and dimerization domains is another small sheet formed by strands k, l and the C-terminal part of D_0 . It lies opposite the gfeah sheet. Their polar interface is occupied largely by hydrophilic side chains and by bound water molecules, in contrast to the tight hydrophobic packing of the helices against the far side of the gfeah sheet.

Domain III. The C-terminal domain of the sE dimer (residues 303–395) has an IgC-like fold¹² (Fig. 2*b*, bottom). It is joined to the central domain through a connecting segment of about 15 residues and anchored to the end of that segment by a disulphide bridge. The axis of the immunoglobulin β -barrel would be perpendicular to the surface of the virus, and its tip would project farther than any other part of the sE dimer. The ABED sheet faces domain I and also contacts the cd-loop of domain II from the other subunit (see Fig. 3*b*). The CFG sheet forms the outer lateral surface of the dimer (Fig. 4). The

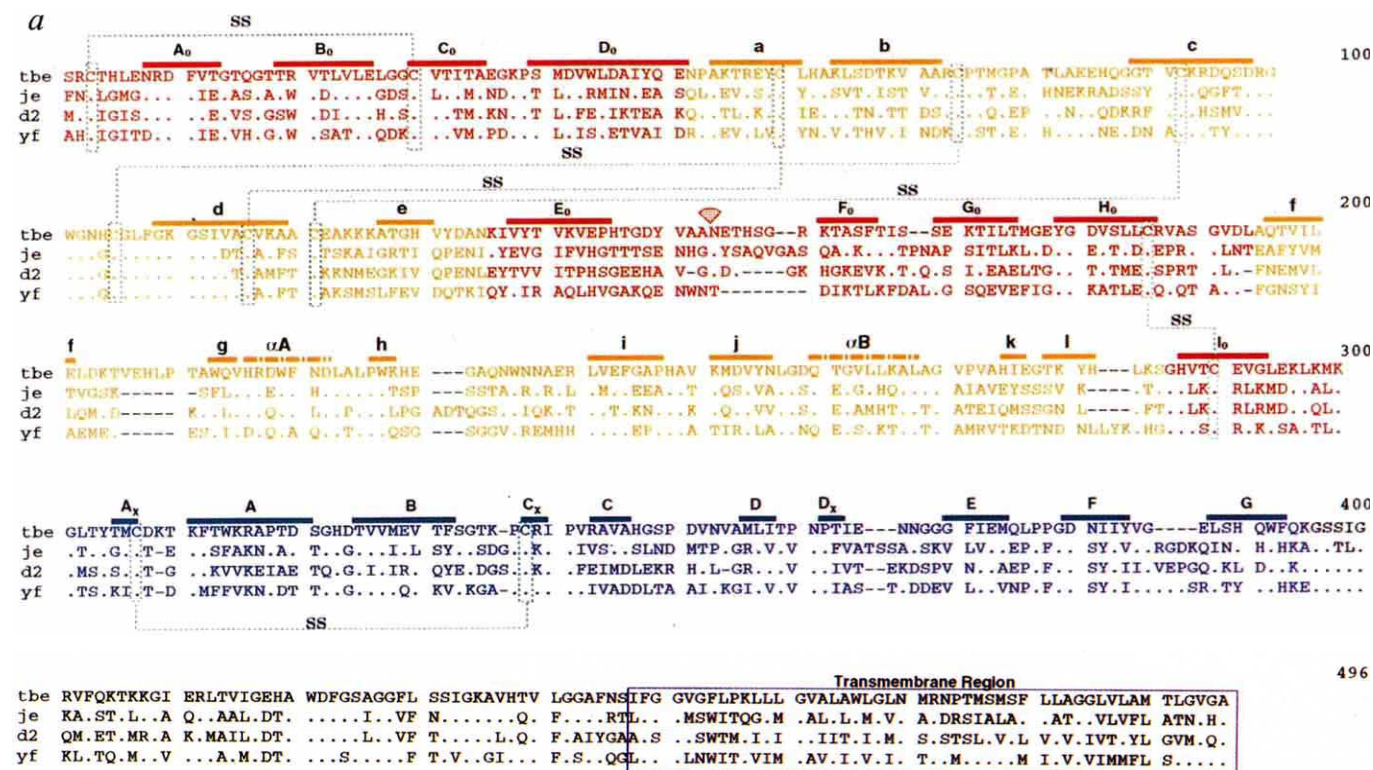


FIG. 2 *a*, Alignment of amino-acid sequences of flavivirus envelope proteins. One representative of each of the four major human pathogenic serotypes is shown: tick-borne encephalitis virus, strain Neudörf (tbe)²⁹; Japanese encephalitis virus, strain JaOArS982 (je)³⁰; yellow fever virus, strain 17D-204 (yf)³¹; dengue virus type 2, strain PR-159 (d2)³². Coloured dots indicate amino-acid identities; black dashes show gaps. The disulphide bridges, assigned chemically for West Nile virus¹³ and confirmed structurally here for TBE virus, are shown by dotted green lines. The domains are colour-coded as in Fig. 1. The C-terminal region that is not part of the soluble fragment is in black; the transmembrane segment is boxed. The glycosylation site on TBE virus is indicated by a red 'fan' above the sequence. We use upper-case letters with subscript 0 (A₀–I₀) for strands in domain I; lower-case letters (a–i) for strands in domain II, upper-case letters (A–G) for strands in domain III. The elements of secondary structure are shown by labelled solid lines (β -strands) and broken lines (α -helices) above the sequence. *b*, Folding diagram for the TBE-sE subunit, together with ribbon representations (drawn with MOLSCRIPT³³) for the individual domains. Each domain contains two apposed layers of secondary structure, which are shown in the folding diagram (left) using distinct shades of the colour for the domain as defined in Fig. 1 and as used in the ribbon diagrams on the right. Disulphide bridges are shown as broken black lines in the folding

diagram and as ball-and-stick cystines in the ribbon diagrams. In domain I, strands A₀C₀D₀E₀F₀ form the 'front' sheet in the folding diagram, and strands B₀I₀H₀G₀ form the 'back' sheet. The view in the ribbon diagram is about the same as in Fig. 1*a*. The three-dimensional structure of this domain differs from other up-and-down β -barrels so far described: the strands on opposite sheets cross at about 40 degrees (rather than 90 degrees), and the barrel is open on one edge. There is no cavity in its interior, as there is in retinol binding protein and other β -barrel calycin domains that bind hydrophobic molecules³⁴. The E₀F₀ loop contains the unique glycosylation site in TBE-E. The Asn-Glu-Thr sequence (residues 154–156) bears a complex oligosaccharide. The two *N*-acetyl-glucosamines (GlcNAc) of its core can be seen in the electron density, and there is also some indication of a fucose linked to O6 of the first GlcNAc. In domain II, sheet gfeah packs against helices α A and α B on one side and faces sheet kId₀ on the other; the tip of the domain is formed by apposed sheets bdc and ij. Strand b lies at the external surface of the virus particle, and on it some flaviviruses carry a second glycosylation site. The view in the ribbon diagram is again the same as in Fig. 1*a*. In domain III, sheets GFC and ABED form an IgC-like module, with a small additional sheet (A_XC_XD_X) at one edge. There is no inter-sheet disulphide in this domain. The view in the ribbon diagram is similar to the left-hand side of Fig. 1*b*.

F and G strands are shorter than those of antibody IgC domains, and the FG loop is abbreviated. The top of the barrel is therefore closed, not by the FG loop, but rather by a small 'extra' sheet (A_XC_XD_X).

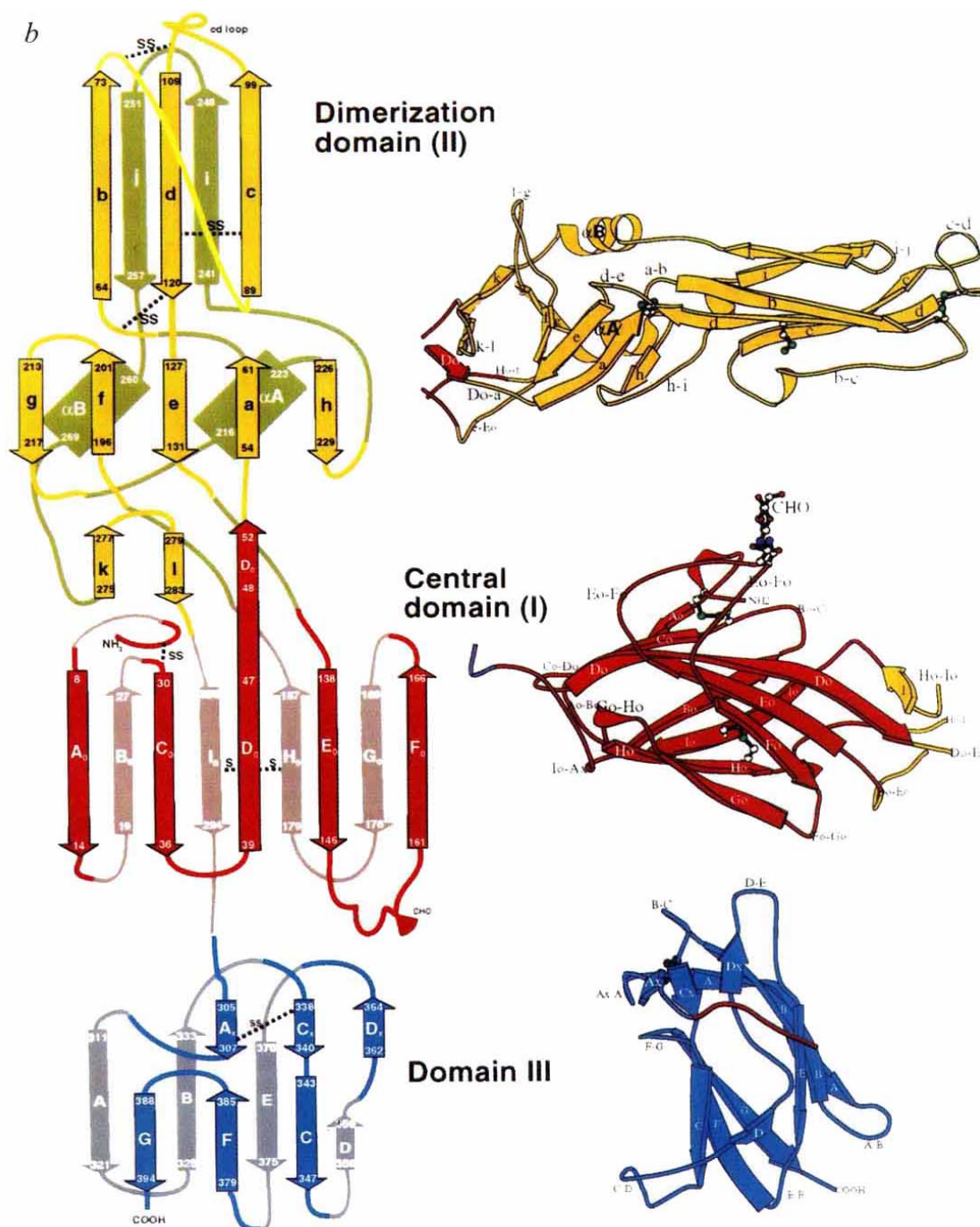
Dimer contacts

The subunit shape leads to an extended, but interrupted, dimer contact, so that there are 'holes' in the dimer (about 20 Å in diameter) on either side of the 2-fold axis (Fig. 1*a*). The dyad-proximal contact contains most polar interactions and van der Waals contacts between hydrophilic side chains. The distal contact is largely non-polar: the conserved cd loop, with a projecting tryptophan, fits into a hydrophobic crevice where domains I and III of the opposite subunit come together (Fig. 3). This interaction is further protected by a 'cover' provided by the oligosaccharide attached to domain I (Fig. 3). It lies just over the groove that receives the cd loop, and it is likely to have a role in stabilizing the dimer. The intrasubunit domain I/domain

III interface is partly hydrophobic, partly polar. Domain III has slightly different orientations, with respect to domains I and II, in the two crystal forms we have used to determine the structure, showing that the interdomain linkage is flexible and that the domains can rock somewhat about their contact.

Other flaviviruses

Figure 2*a* shows an alignment of E-protein sequences from representatives of the four major human-pathogenic serogroups of flaviviruses. The overall amino-acid identity between any two is about 40 per cent⁶. All 12 cysteine residues are conserved. These form six disulphide bridges that have been assigned for West Nile virus (a member of the JE subgroup)¹³, and all six are present in our structure of TBE-sE. The model also accounts for regions of particularly high sequence variation among the groups, for example, in the E₀F₀ and fg loops. Thus, the model can be taken to show the folded structure of all flavivirus E proteins, and in analysing properties such as antigenicity, low-



pH conformational change and fusogenic activity, we can map results from various flaviviruses onto it.

Antigenic sites

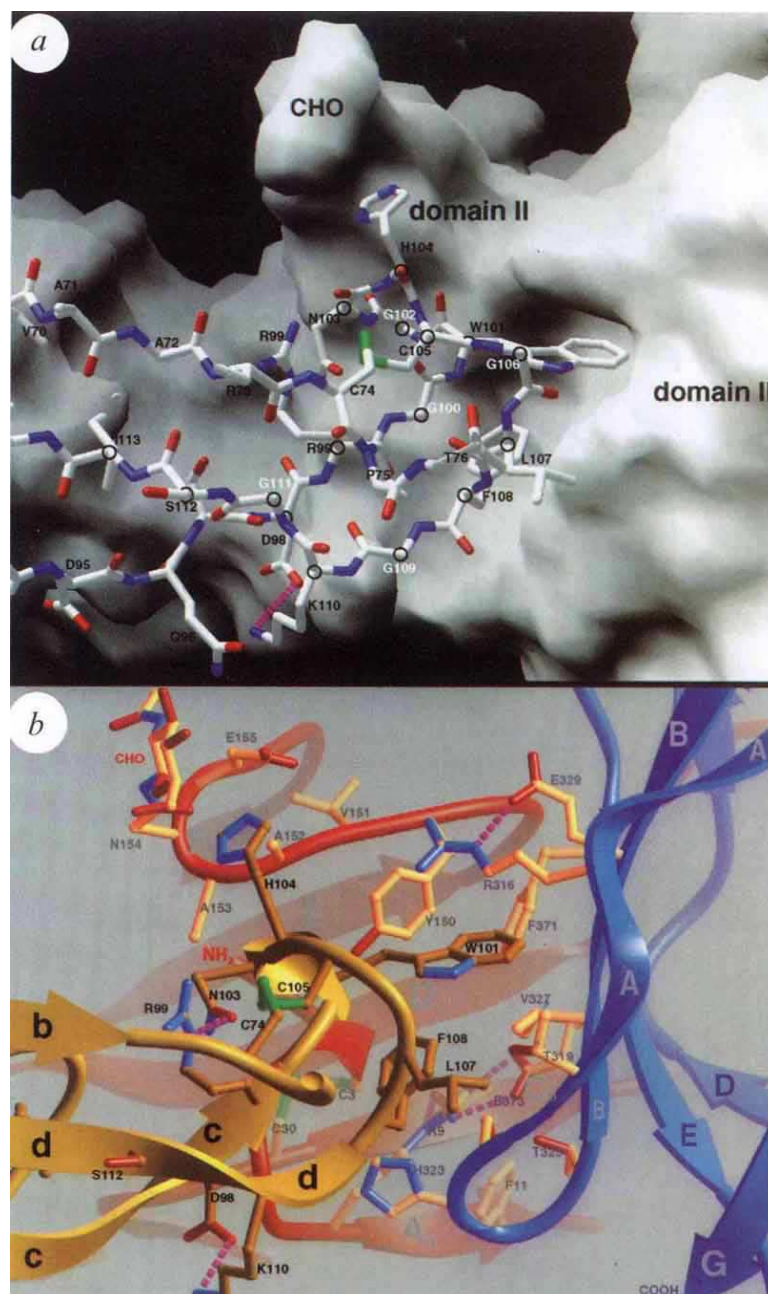
A model for the antigenic structure of the TBE virus E protein has been proposed¹⁴, which maps many of the known epitopes into three antigenic domains, called A, B and C. These correspond well to domains II, III and I, respectively, in the three-dimensional structure. Flavivirus crossreactive epitopes have been assigned to domain A, whereas only type- or subtype-specific epitopes have been found in the other domains^{14,15}. Figure 5a shows mutation sites that abolish binding of the corresponding neutralizing antibodies on TBE virus. Most lie on ridges on the convex face, consistent with our interpretation of external and

internal molecular surfaces. The exceptions are B1 and B4, both on domain III, which lie on the lateral surface of the CFG sheet. On the virion, this face must therefore be accessible to antibodies.

The low-pH conformational change

What can we infer about the conformational change that is triggered by exposure to low pH? It is clear that the entire dimer interface must come apart, because the state of association on the viral surface switches from dimer to trimer⁷. A mutation that eliminates the glycosylation site in dengue 2 virus leads to a higher pH threshold¹⁶, consistent with participation of the oligosaccharide in the dimer contact as seen in our structure (Fig. 4a, b). The low-pH conformational change cannot, however, be

FIG. 3 The contact between the cd loop of domain II (proposed 'fusion peptide'¹¹) on one subunit (foreground) and the domain I/domain III interface on the other (background), viewed as on the right-hand side of Fig. 1b. *a*, The background subunit is shown in a surface representation. It forms a hydrophobic pocket at the interface of domains I and III, into which aromatic side chains from the cd loop of the foreground subunit fit snugly (Trp 101 and Phe 108). The open circles mark the positions of the α -carbons of residues 98–113, conserved in nearly all flaviviruses, which span the cd loop. The carbohydrate (CHO) covers this hydrophobic contact on the outer surface of the molecule. The complete oligosaccharide is much larger than the two-sugar segment modelled here. Drawn with GRASP³⁵. *b*, The same contact in a ribbon representation, colour-coded as in Fig. 1, with side chains drawn in orange for the foreground subunit (cd loop) and in pale yellow for the background subunit (domain I/domain III interface). Hydrogen bonds are shown as broken cylinders (magenta). Phe 108 and Trp 101 of one subunit can be seen to pack against Tyr 150 (domain I), Phe 371 and Val 327 (domain III) of the other. This hydrophobic contact is covered, on the outer surface of the molecule, by the salt bridge between Arg 316 and Glu 329. The interface between domains I and III is further stabilized by the salt bridge between Arg 9 and Glu 373, residues that are strictly conserved in all flaviviruses. His 104 packs against the first sugar residue (*N*-acetyl-glucosamine) of the carbohydrate, which is attached to Asn 154 of domain I. The conformation of the cd loop is held in place by the disulphide between Cys 74 and Cys 105, shown in green. Drawn with RIBBONS²⁸.



induced in the free sE dimer (F.X.H., unpublished observations), indicating that the driving force must come in part from other changes in the E-protein network on the surface of the virion; for example, from rearrangements in the 46-residue region between the C terminus of the sE fragment and the transmembrane segment. It is possible that this intervening region participates in trimer contacts.

As summarized in Fig. 5a, the binding of certain monoclonal antibodies is strongly affected by the conformational switch⁸. Most of the epitopes in domain II are no longer recognized, suggesting that when the dimer contact is broken, a significant reorganization occurs either in the tertiary structure of domain II, or in its association with other domains or other subunits. Antibodies against synthetic peptides derived from sequences spanning the domain II of dengue 2 envelope protein are selectively reactive with low-pH-treated virus and exhibit little or no binding to mature virus at neutral pH¹⁷, indicating that there is substantially increased exposure of the domain in the low-pH

form. Antibodies that recognize the external edge of the central domain (antibodies i2 and IC3, Fig. 5b) also bind poorly after the low-pH rearrangement, suggesting further changes in contacts within the surface of the virion. By contrast, binding of antibodies to the C-terminal domain is much less affected.

Domain II is highly crosslinked by disulphide bonds, which may restrict internal structural changes. Its altered antigenicity after the low-pH transition therefore suggests that it might swing outwards to expose additional surface. The base of the domain indeed has hinge-like characteristics, particularly the loosely packed polar interface between the short gfeah and kID₀ sheets. A hinge motion about this region would elevate the tip of the domain above the viral membrane and bring the cd loop into prominence. The strict conservation and hydrophobic character of this loop support the notion that it participates in membrane fusion. It is deeply buried in the native dimer (Fig. 3), but the residues in domain I and III with which it interacts are more variable, indicating that the characteristics of this contact are

FIG. 4 The lateral surface of domain III, viewed along an axis perpendicular to the two images shown in Fig. 1. This surface contains residues implicated as determinants of host range, tropism or virulence in different flaviviruses. As described in the text, we propose that this surface may be involved in receptor recognition. *a*, Ribbon representation of domain III with all side chains. The underlined residues are the sites of mutations affecting one or more of the properties mentioned above. Mutation of the RGD sequence in Murray Valley encephalitis virus (in the 4-residue insert before TBE residue 387) leads to reduced neurovirulence in mice³⁶. A tyrosine-to-histidine mutation at position 384 in TBE virus also leads to attenuation³⁷, as do separate mutations at residues 308 and 310 in louping ill virus (also tick-borne)^{38,39} and one at position 335 in JE⁴⁰. The potential significance of residue 311 in yellow fever virus is explained in the text. All residue numbers mentioned here refer to the alignment with TBE displayed in Fig. 2a. *b*, A surface representation, coloured according to the electrostatic potential, of domain III. Negative potential is represented in red and positive in blue. The residues described above form the prominent features of the molecular surface at the ends of the dimer. Drawn with GRASP³⁵.

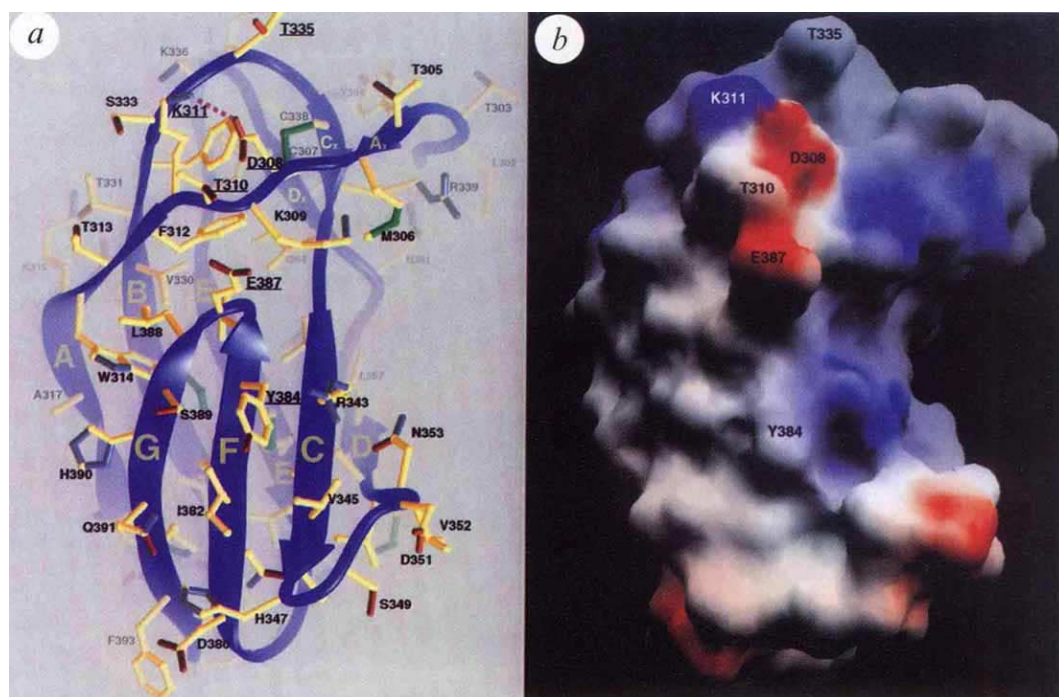
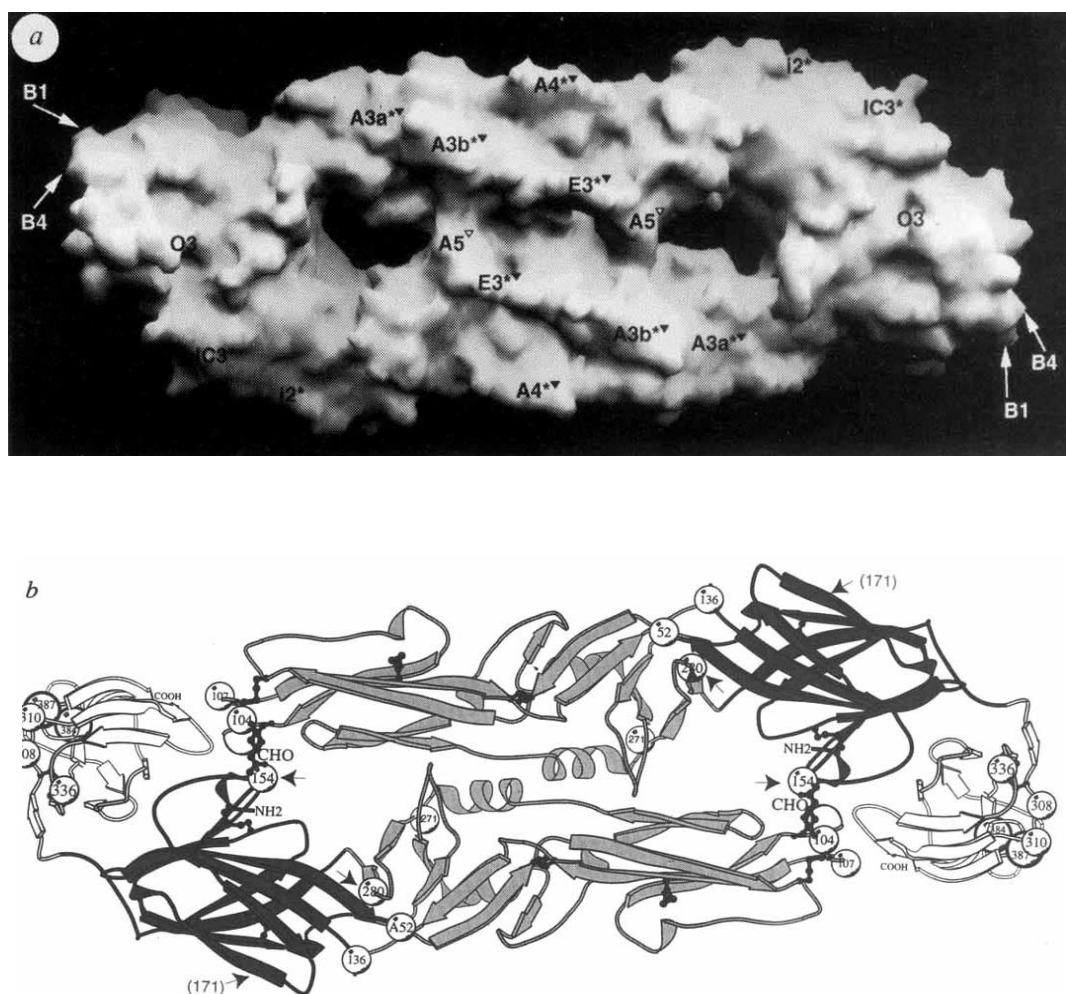


FIG. 5 *a*, Locations of the mutations that affect binding of neutralizing monoclonal antibodies to epitopes on the TBE envelope glycoprotein, mapped onto a surface representation of the dimer. The sites are labelled by their published designations^{14,41}. The symbols for the individual epitopes are coded to show the effect of the low-pH conformational change or of the presence of prM on immature virions⁸. Triangles indicate sites masked (solid) or enhanced (open) by prM. Stars mark sites where binding of monoclonal antibodies is diminished by low-pH treatment. Drawn with GRASP³⁵. *b*, Sites of mutations from several flaviviruses that affect virulence. As described in the text, these mutations cluster into three regions on the three-dimensional structure. Three sites where mutations influence the threshold pH for the fusion-activating conformational change are indicated by arrows. Mutations at positions 52 (ref. 42), 136 (H. Sumiyochi, personal communication), 271 and 336 (ref. 40) are from JE; those at positions 280 (P. C. McMinn, personal communication) and 387 (ref. 36), from Murray Valley encephalitis virus, at 104 and 107, from a TBE/Den 4 chimera⁴³; at 308 and 310, from louping ill virus^{38,39}, at 171 (ref. 14) and 384 (ref. 37) from TBE virus and at 154, the glycosylation site, from a TBE/Den 4 chimera⁴³, from Den 2 (ref. 16), and from Den 4 (ref. 44). All numbers refer to the positions in TBE, Neudörfli strain (Fig. 2a). Drawn with MOLSCRIPT³³.



not the basis for its conservation. Moreover, the dimer-to-trimer transition in the fusion-activating conformational change⁷ implies that the cd loop becomes exposed, and the hinge motion we propose would project it towards the target cell membrane. Mutations in this hinge region can indeed influence virulence and the transition pH (Fig. 5*b*). Dramatic outward projection of a previously buried fusion peptide has been demonstrated in the functionally related but structurally different case of influenza HA¹⁸.

Immature virions, which contain the uncleaved prM protein, do not undergo the low-pH rearrangements⁸. Crosslinking and sedimentation experiments show that prM and E are closely associated, probably as a heterodimer^{8,19}. Figure 5a shows antigenic sites that are unreactive in immature virions. The protected sites are restricted to domain II, suggesting that prM contacts this part of E and stabilizes the dimeric, non-fusogenic conformation.

Determinants of virulence and tropism

Viral surface proteins are generally major determinants of host range and cell tropism and often of virulence or attenuation. Single mutations in the E protein of a number of flavivirus strains are responsible for changes in these properties; their locations are displayed in Fig. 5*b* and the sources are cited in the legend. The sites cluster in three distinct regions: (1) the distal face of domain III (sheet CFG); (2) the base of domain II (between sheets gfeah and kID₀); (3) the contact between the domain I/domain III interface and the cd loop of the opposite subunit. Changes in the last two regions are likely to influence virulence by affecting the low-pH conformational transition; indeed, mutations that alter the critical pH for this transition also map to the same or neighbouring sites (Fig. 5*b*). In contrast, it is cell attachment that is likely to be affected by mutations on the distal face of domain III, where binding of monoclonal

antibodies is much less influenced by the low-pH transition. This group of sites is centred about the FG loop (Fig. 4), which is a tight turn in TBE and other tick-borne flaviviruses but which contains four additional residues in all mosquito-borne strains. Because the viruses replicate in their vectors, this loop is likely to be a host-range determinant. In many of the mosquito-borne viruses, the enlarged FG loop contains an Arg-Gly-Asp (RGD) sequence, the motif recognized by integrins such as the fibronectin type III receptor²⁰. Mutations affecting virulence or cell tropism also cluster in this region (see legend to Fig. 4). Particularly interesting is a rare revertant of the yellow fever (17D) vaccine strain, isolated from a patient with a fatal encephalitis, in which mutations at residues 155 and 311 (TBE numbering) appear to have produced a neurotropic (rather than viscerotropic) virus²¹. Domain III is, to our knowledge, the first example of an immunoglobulin-module in a viral surface protein. (Such a domain has recently been found on the outer surface of a non-enveloped insect virus: J. Johnson, personal communication.) The widespread occurrence of such structures in cell-adhesion proteins is consonant with the suggestion that this domain participates in attachment to a cellular receptor.

Analysis of the mutations just described show that the structure provides the framework for a detailed understanding of specific mechanisms of the viral life cycle and their role in the biology of flaviviruses. In particular, it suggests that it should be possible to engineer attenuated strains as starting points for vaccines. The YF vaccine developed by Theiler and Smith²² was the first successful use of a live, attenuated viral strain to confer long-term immune protection against the virulent parent strain from which it was derived. The attenuated virus was obtained by extensively repeated passage of YF through chick embryonic tissue. Suitable live vaccines for dengue, TBE, JE and other flaviviruses are still being sought. Understanding the mechanisms affected by attenuating mutations in the E protein should inform and accelerate the development of such strains. □

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1. Wilson, I. A., Skehel, J. J. & Wiley, D. C. *Nature* **289**, 366–373 (1981).
2. Varghese, J. N., Laver, W. G. & Colman, P. M. *Nature* **303**, 35–40 (1983).
3. Heinz, F. X. et al. *J. Virol.* **65**, 5579–5583 (1991).
4. Monath, T. P. in *Virology* (eds Fields, B. N. & Knipe, D. M.) 763–814 (Raven, New York, 1990).
5. Chambers, T. J., Hanh, C. S., Galler, R. & Rice, C. M. *A. Rev. Microbiol.* **44**, 649–688 (1990).
6. Heinz, F. X. & Roehrig, J. T. in *Immunocytochemistry of Viruses Vol. 2 The Basis for Serodiagnosis and Vaccines* (eds van Regenmortel, M. H. V. & Neurath, A. R.) 289–305 (Elsevier Science (Biomedical Division), Amsterdam, 1990).
7. Allison, S. L. et al. *J. Virol.* **69**, 695–700 (1995).
8. Heinz, F. X. et al. *Virology* **198**, 109–117 (1994).
9. Guirakhoo, F., Heinz, F. X., Mandl, C. W., Holzmann, H. & Kunz, C. *J. gen. Virol.* **72**, 1323–1329 (1991).
10. Guirakhoo, F., Bolin, R. A. & Roehrig, J. T. *Virology* **191**, 921–931 (1992).
11. Roehrig, J. T., Hunt, A. R., Johnson, A. J. & Hawkes, R. A. *Virology* **171**, 49–60 (1989).
12. Bork, P., Holm, L. & Sander, C. *J. molec. Biol.* **242**, 309–320 (1994).
13. Nowak, T. & Wengler, G. *Virology* **156**, 127–137 (1987).
14. Mandl, C. W., Guirakhoo, F., Holzmann, H., Heinz, F. X. & Kunz, C. *J. Virol.* **63**, 564–571 (1989).
15. Heinz, F. X. *Adv. Virus Res.* **31**, 103–168 (1986).
16. Guirakhoo, F., Hunt, A. R., Lewis, J. G. & Roehrig, J. T. *Virology* **194**, 219–223 (1993).
17. Roehrig, J. T., Johnson, A. J., Hunt, A. R., Bolin, R. A. & Chu, M. C. *Virology* **177**, 668–675 (1990).
18. Bullough, P. A., Hughson, F. M., Skehel, J. J. & Wiley, D. C. *Nature* **371**, 37–43 (1994).
19. Wengler, G. & Wengler, G. *J. Virol.* **63**, 2521–2526 (1989).
20. Ruoslahti, E. & Pierschbacher, M. D. *Science* **238**, 491–497 (1987).
21. Jennings, A. D. et al. *J. infect. Dis.* **169**, 512–518 (1994).
22. Theiler, M. & Smith, H. H. *J. exp. Med.* **65**, 787–800 (1937).
23. Blum, M. L. thesis, Harvard Univ. (1990).
24. Bricogne, G. *Acta crystallogr.* **A32**, 832–847 (1976).
25. Jones, T. A., Zhou, J.-Y., Cowan, S. W. & Kjeldgaard, M. *Acta crystallogr.* **A47**, 110–119 (1991).

26. Brünger, A. T. *X-PLOR Version 3.1.A System for X-ray Crystallography and NMR* (Yale University Press, New Haven, CT, 1992).
27. Brünger, A. T. *Nature* **355**, 472-475 (1992).
28. Carson, M. *J. mole. Graph.* **5**, 103-106 (1987).
29. Mandl, C. W., Heinz, F. X. & Kunz, C. *Virology* **166**, 197-205 (1988).
30. Sumiyoshi, H. et al. *Virology* **161**, 497-510 (1987).
31. Rice, C. M. et al. *Science* **229**, 726-733 (1985).
32. Lewis, J. A. et al. *Virology* **197**, 216-224 (1993).
33. Kraulis, P. E. *J. appl. Crystallogr.* **24**, 946-956 (1991).
34. Flower, D. R., North, A. C. T. & Attwood, T. K. *Protein Sci.* **2**, 753-761 (1993).
35. Nichols, A., Bharadwaj, R. & Honig, B. *Biophys. J.* **64**, A166-A166 (1993).
36. Lobigs, M. et al. *Virology* **176**, 587-595 (1990).
37. Holzmänn, H., Heinz, F. X., Mandl, C. W., Guirakhoo, F. & Kunz, C. *J. Virol.* **64**, 5156-5159 (1990).
38. Jiang, W. R., Lowe, A., Higgs, S., Reid, H. & Gould, E. A. *J. gen. Virol.* **74**, 931-935 (1993).
39. Gao, G. F., Hussain, M. H., Reid, H. W. & Gould, E. A. *J. gen. Virol.* **75**, 609-614 (1994).
40. Cecilia, D. & Gould, E. A. *Virology* **181**, 70-77 (1991).
41. Holzmänn, H. et al. *Arch. Virol.* **140**, 213-221 (1995).
42. Hasegawa, H., Yoshida, M., Shiosaka, T., Fujita, S. & Kobayashi, Y. *Virology* **191**, 158-165 (1992).
43. Pletnev, A. G., Bray, M. & Lai, C.-J. *J. Virol.* **67**, 4956-4963 (1993).
44. Kawano, H., Rostapshov, V., Rosen, L. & Lai, C.-J. *J. Virol.* **67**, 6567-6575 (1993).

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Prospects for detection of extra-solar giant planets by next-generation telescopes

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THE construction of several large ground-based telescopes^{1,2} and the anticipated launches of new space-based ones³⁻⁵, have prompted renewed interest in the means by which extra-solar planets might be discovered^{1,6,7-11}. The direct detection of light from such a planet would be the most compelling means of discovery, and it may soon be technically feasible^{1,6}. Jupiter has traditionally been used as a benchmark for observability, but extra-solar giant planets could have a wide range of masses and ages¹², and could be significantly brighter than Jupiter. Here we present calculations estimating the optical and infrared fluxes of extra-solar giant planets with a range of ages, and demonstrate the conditions under which they will be observable with several new telescopes. Giant planets with masses greater than that of Jupiter, and younger than about 1 billion years, are the best targets, and they should be visible using the generation of telescopes now under construction.

Extra-solar giant planets (EGPs) will radiate at optical wavelengths by reflection and in the infrared by the thermal emission of both absorbed stellar light and the planet's own internal energy. To calculate their cooling curves, we used the Henyey code previously constructed to study brown dwarfs and M dwarfs^{13,14}. Below effective temperatures (T_{eff}) of 600 K, we employed the model atmospheres of Graboske *et al.*¹⁵, who included opacities due to water, methane, ammonia and collision-induced absorption by H_2 and He. The gravity dependence of the EGP atmospheres was modelled as described by Hubbard¹⁶. Above $T_{\text{eff}} = 600$ K, we used the X model of ref. 14. The two models were interpolated in the overlap region. We employed the hydrogen/helium equation of state of Saumon and Chabrier^{17,18} and ignored rotation and the possible presence of an ice/rock core¹⁹⁻²⁰. The EGPs were assumed to be fully convective at all times. We included the effect of 'insolation' by a central star of mass M_* and considered semimajor axes (a) between 2.5 and 20 AU. Giant planets may form preferentially near 5 AU (ref. 21), but a range of a values cannot be excluded. We assumed that the Bond albedo of an EGP is that of Jupiter (0.35; ref. 22). For this study, we evolved EGPs with masses (M_p) from $0.3 M_J$ (the mass of Saturn) to $15 M_J$ ($M_J \approx 1.9 \times 10^{30}$ g, where M_J is the mass of Jupiter). Whether a $15 M_J$ object is a planet or a brown dwarf is largely a semantic issue, though one might distinguish gas giants and brown dwarfs by their mode of formation (for example, in a disk or 'directly'). Physically, compact hydrogen-rich objects with masses from $0.00025 M_\odot$ to $0.25 M_\odot$ form a continuum. However, our EGPs above $\sim 13 M_J$ do burn 'primordial' deuterium for up to 10^8 years. Note that any search for giant planets will be even more capable of discovering brown dwarfs.

The evolution of the bolometric luminosity (L_{bol}) of our suite of EGPs orbiting 5.2 AU from a G2V star is depicted in Fig. 1. The high values of L_{bol} for early ages and high masses are striking. That a young 'Jupiter' or 'Saturn' will be bright has been known for some time^{15,16,19,20,23-26}, but ours are the first detailed calculations for objects with $M_p > M_J$ and ages, t , greater than 10^7 years. Below $\sim 10 M_J$, L_{bol} is very roughly proportional to

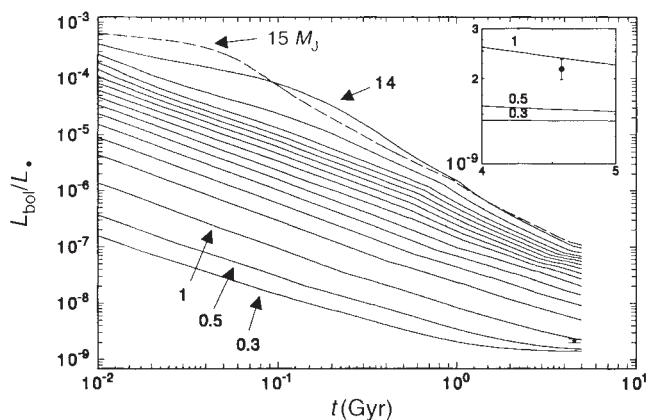


FIG. 1 Bolometric luminosity (L_{bol}) in solar units of a suite of EGPs located at a distance of 5.2 AU from a G2V star, plotted against time (t) in Gyr. The reflected luminosity is not included, but the absorbed component is. At $t \approx 0.2$ Gyr, the luminosity of the $14 M_J$ EGP exceeds that of the $15 M_J$ EGP because of late deuterium ignition. The data point at 4.55 Gyr shows the observed luminosity of Jupiter²⁶. The $0.3 M_J$ EGP exhibits a strong effect of warming by the G2V primary star at late stages in its evolution. Although this model resembles Saturn in mass, here it is placed at the distance of Jupiter from its primary. (The flattening in L versus t for low masses and great ages is a consequence of stellar insolation.) The inset shows, on an expanded scale, the comparison of our lowest-mass evolutionary trajectories with the present Jupiter luminosity.

M_p^α / t^β , where $1.6 \leq \alpha \leq 2.1$ and $1.0 \leq \beta \leq 1.3$. An EGP with a mass of $2 M_J$ at age 10^7 years is 2,000 times brighter than the current Jupiter (and its T_{eff} is ~ 700 K). At the age of the Pleiades ($\sim 7 \times 10^7$ yr), such an EGP would be ~ 200 times brighter (with $T_{\text{eff}} \approx 420$ K) and at the age of the Hyades ($\sim 6 \times 10^8$ yr) it would be ~ 18 times brighter (with $T_{\text{eff}} \approx 235$ K). The measured L_{bol} and T_{eff} of Jupiter are $(2.186 \pm 0.022) \times 10^{-9} L_\odot$ and 124.4 ± 0.3 K, respectively²⁶. At an age of 4.55 Gyr, our model of Jupiter has a luminosity of $2.35 \times 10^{-9} L_\odot$ and an effective temperature of 122 K.

A complete discussion of our results will be reported elsewhere (D. S. *et al.*, manuscript in preparation). We mention here a few selected outcomes of our model to illustrate the character of massive, young EGPs. As the fluxes at wavelengths $< 10 \mu\text{m}$ (the N band) are generally on or near the Wien tail of the EGP spectrum, the fluxes in the near- and mid-infrared spectral bands increase even faster with mass and youth than L_{bol} . In particular, assuming that the emission is planckian, that the orbital separation is 5.2 AU, and that $M_* = 1.0 M_\odot$, Jupiter's N-band flux would be $\sim 8,000$ times higher at age 10^7 years than it is now. At the age of our Solar System, a $2 M_J$ EGP and a $5 M_J$ EGP would be ~ 6 and ~ 90 times brighter in the N band than the current Jupiter. Furthermore, in the M band ($\sim 5 \mu\text{m}$) a $2 M_J$ EGP would be $\sim 60,000$ times brighter at 10^7 years than the current Jupiter, but at 10^9 years 'only' ~ 2.5 times brighter than a coeval Jupiter. At the age of the Hyades, Saturn would be as bright as the current Jupiter. The fluxes at Earth due to thermal emissions at wavelengths $< 10 \mu\text{m}$ of EGPs in the Pleiades (distance $D \approx 125$ pc) would be greater than those from EGPs in the Hyades ($D \approx 45$ pc), despite the latter's relative proximity, because the Pleiades are younger (and, hence, at higher T_{eff}).

Here we focus on the crucial question of the brightness of EGPs as a function of age and mass to aid in the development of search strategies. However, the detection of EGPs requires telescopes with both high sensitivity and high angular resolution. The latter is necessary to discriminate the planet from the star, and can be compromised by the presence of scattered light in the telescope optics. Nevertheless, it is expected that the Large Binocular Telescope¹ (LBT), the Near Infrared Camera and

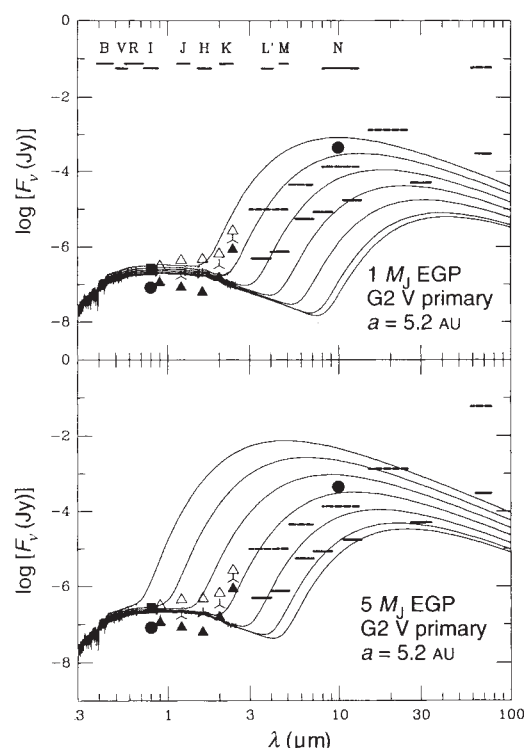


FIG. 2 Wavelength dependence of the flux received at the Earth from extra-solar giant planets (EGPs) orbiting at 5.2 AU from a G2V star at 10 pc from the Earth (the orbit subtends an angle of ~ 0.5 arcsec). Objects of $1 M_J$ (top panel) and $5 M_J$ (bottom panel) are displayed at the following ages: $\log t(\text{yr}) = 7.0, 7.5, 8.0, 8.5, 9.0, 9.5$ and 9.7 (from left to right). The reflected component of the light is essentially independent of the mass and the age of the EGP. The EGP is assumed to emit like a black body and to reflect incident light as a grey body. Standard photometric bandpasses are shown at the top. Also shown are the design sensitivities of several astronomical systems for the detection of point sources with a signal-to-noise ratio of 5 in a 1-h integration (40 min for NICMOS). These systems are the LBT and MMT (solid circles and square, respectively), the three cameras of NICMOS (open triangles, three-pointed stars and solid triangles), SIRTf (solid bars) and Gemini and SOFIA (dashed bars). The spectrum of a G2V star was provided by A. Eibl (personal communication). It should be stressed that although SIRTf is unlikely to have the angular resolution to detect the EGPs of this example, the same EGPs at slightly larger separations around a star that is slightly closer will be well within its detection envelope (see Fig. 3 legend).

Multi-Object Spectrometer³ (NICMOS) and the Space Infrared Telescope Facility⁴ (SIRTf) will have both the sensitivity and the angular resolution (see Fig. 3 legend) to discover EGPs with a variety of realistic combinations of a and D . It is not our purpose here to discuss various detection strategies, nor to explore the consequences of every combination of M_p , M_* , a , D and telescope. Rather, in Figs 2 and 3, we compare theoretically predicted fluxes for a few representative values of a and D and various EGP ages and masses with the flux sensitivities of various ground- and space-based telescopes currently being developed.

Figure 2 depicts the flux in Janskys versus wavelength (at times between 10^7 and 5×10^9 yr) for a $1 M_J$ and a $5 M_J$ EGP that are 5.2 AU from a G2V star (a solar analogue) that is 10 pc from the Earth. We have made the assumption that the thermal emissions are black-body and have included the reflected light. Also shown on Fig. 2 are the 5σ point-source sensitivities at various wavelengths for SIRTf³, the LBT¹, the upgraded 'Multiple Mirror' Telescope¹ (MMT), Gemini², the Stratospheric Observatory For Infrared Astronomy²⁷ (SOFIA) and the three NICMOS cameras³. As is indicated in Fig. 2, the LBT and

NICMOS have the flux sensitivity to see at 10 pc the reflected light of such EGPs at any age. At the diffraction limit, these instruments will also have the requisite angular resolution. At 10 pc, SIRTf has the flux sensitivity between $5 \mu\text{m}$ and $10 \mu\text{m}$ to detect the thermal emissions of both a $5 M_J$ EGP, for ages $< 10^9$ years, and a Jupiter at 10 AU, for ages $< 10^8$ years. Figure 3 shows the fluxes from EGPs with masses between $0.3 M_J$ and $5 M_J$ orbiting at 10 AU around an A0V star (for example, a Vega analogue) at a distance of 10 pc from the Earth. Its age is 2×10^8 yr, just less than its main-sequence lifetime. It can be seen that the reflected light from all EGPs at 10 AU around such a bright star will be detectable by the LBT, the MMT and NICMOS, because all these telescopes will achieve angular resolutions well below 1 arcsec. Furthermore, the SIRTf sensitivities in the mid-infrared would be just adequate to see even a Saturn-mass object around an A0V star at this age, distance and separation. Although SIRTf may not achieve the necessary 1-arcsec performance, it should have the angular resolution to discover similar EGPs at somewhat smaller D s and larger a s.

Interestingly, NICMOS is sensitive enough to detect any widely-separated EGP with a mass greater than $6 M_J$ around any main-sequence star as far away as the Pleiades, whereas SIRTf will have the sensitivity to detect any 'free-floating EGPs' in the Pleiades with a mass $> 4 M_J$. This is because the internal energy of a young EGP alone is enough to power it in the relevant spectral bands. None of the anticipated telescope systems will have the angular resolution to probe for an EGP in the Pleiades, unless it is many tens of astronomical units from the central star.

Searches around early main-sequence stars or in very young stellar systems will select for the youngest and most massive planets. Crude models of giant-planet formation around a solar-mass star permit objects up to $10 M_J$ to form¹². The most massive

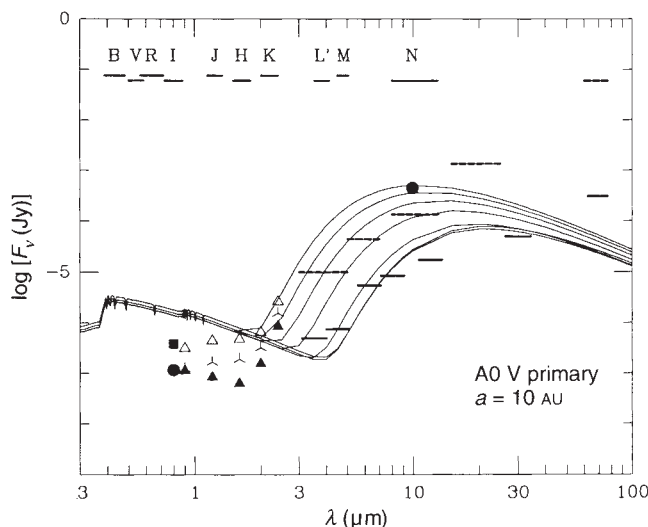


FIG. 3 As Fig. 2, but for EGPs orbiting 10 AU from an A0V star whose age is 2.0×10^8 yr and whose distance is 10 pc. (The angular separation is 1 arcsec.) From left to right, the curves correspond to masses of 5, 4, 3, 2, 1, 0.5 and $0.3 M_J$. The spectrum of an A0V star is from Dreiling and Bell³¹. Note that the heating by this star at 10 AU is quite significant for all lower-mass EGPs and that the reflected component of an EGP is ~ 80 times brighter when it orbits an A0V star than when it orbits a G2V star. The MMT, the LBT and NICMOS (on the Hubble Space Telescope), with apertures of 6.5 m, 8.4 m ($\times 2$), and 2.4 m, respectively, will achieve angular resolutions near their respective diffraction limits (ref. 1, and N. Woolf and G. Schneider, personal communications). Being a mid-infrared space telescope with a 0.85-m aperture, SIRTf's angular resolution will, quite naturally, be poorer than that of the optical and near-infrared space telescopes. But with super-resolution techniques, SIRTf should be able to resolve EGPs at somewhat larger a s and smaller distances than used for this figure (F. Low, personal communication).

planet in our Solar System is Jupiter and why there are none more massive is not understood. The lifetime of our gaseous proto-planetary disk may have been short²⁸ or the disk may have been tidally truncated by the growing planet itself²⁹. Good statistics on the population of EGPs will better constrain proto-planetary disk processes. The absence of a Jupiter-class planet in a planetary system would imply a very different population of cometary-sized planetesimals than exists in our own Solar System, and this may have important implications for the origin, evolution and survival of life on rocky 'terrestrial' planets³⁰. Only sensitive and systematic searches, such as those we anticipate over the next decade, will directly address these important issues of planet formation. □

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1. Angel, R. *Nature* **368**, 203–207 (1994).
2. Mountain, M., Kurz, R. & Oschmann, J. *Proc. SPIE* **2199**, 41–55 (1994).
3. Thompson, R. *Space Sci. Rev.* **61**, 69–93 (1992).
4. Erickson, E. F. & Werner, M. W. *Space Sci. Rev.* **61**, 95–98 (1992).
5. Benvenuti, P. et al. in *ESA's Report to the 30th COSPAR Meeting*, ESA SP-1169 (ed. Burke, W. R.) 78–81 (ISO, Paris, 1994).
6. Burke, B. F. et al. *TOPS: Toward Other Planetary Systems* (NASA Solar System Exploration Division, Washington DC, 1992).
7. Gatewood, G. D. *Astr. J.* **94**, 213–224 (1987).
8. Reasenberg, R. D. et al. *Astr. J.* **96**, 1731–1745 (1988).

9. Walker, G. A. H. et al. *Icarus* (in the press).
10. Mao, S. & Paczynski, B. *Astrophys. J.* **374**, L37–L40 (1991).
11. Borucki, W. J. & Summers, A. L. *Icarus* **58**, 121–134 (1984).
12. Podolak, M., Hubbard, W. B. & Pollack, J. B. in *Protostars and Planets III* (eds Levy, E. & Lunine, J. I.) 1109 (Univ. Arizona Press, Tucson, 1993).
13. Burrows, A., Hubbard, W. B. & Lunine, J. I. *Astrophys. J.* **345**, 939–958 (1989).
14. Burrows, A., Hubbard, W. B., Saumon, D. & Lunine, J. I. *Astrophys. J.* **406**, 158–171 (1993).
15. Graboske, H. C., Pollack, J. B., Grossman, A. S. & Olness, R. J. *Astrophys. J.* **199**, 265–281 (1975).
16. Hubbard, W. B. *Icarus* **30**, 305–310 (1977).
17. Saumon, D. & Chabrier, G. *Phys. Rev. A* **44**, 5122–5141 (1991).
18. Saumon, D. & Chabrier, G. *Phys. Rev. A* **46**, 2084–2100 (1992).
19. Pollack, J. B. A. *Rev. Astr. Astrophys.* **22**, 389–424 (1984).
20. Bodenheimer, P. & Pollack, J. B. *Icarus* **67**, 391–408 (1986).
21. Boss, A. P. *Science* **267**, 360–362 (1995).
22. Conrath, R. A., Hanel, R. A. & Samuelson, R. E. in *Origin and Evolution of Planetary and Satellite Atmospheres* (eds Atreya, S. K., Pollack, J. B. & Matthews, M. S.) 513–538 (Univ. Arizona Press, Tucson, 1989).
23. Saumon, D., Hubbard, W. H., Chabrier, G. & Lunine, J. I. *Astrophys. J.* **391**, 827–831 (1992).
24. Guillot, T., Chabrier, G., Gautier, D. & Morel, P. *Astrophys. J.* (in the press).
25. Black, D. C. *Icarus* **43**, 293–301 (1980).
26. Pearl, J. C. & Conrath, R. A. *J. geophys. Res. Suppl.* **96**, 18921–18930 (1991).
27. Erickson, E. F. *Space Sci. Rev.* **61**, 61–68 (1992).
28. Zuckermann, B., Forveille, T. & Kastner, J. H. *Nature* **373**, 494–496 (1995).
29. Lin, D. N. C. & Papaloizou, J. *Mon. Not. R. astr. Soc.* **186**, 799–812 (1979).
30. Wetherill, G. W. *Lunar Planet. Sci. Conf.* **XXIV**, 1511–1512 (1993).
31. Dreiling, L. A. & Bell, R. A. *Astrophys. J.* **241**, 736–758 (1980).

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Superconductivity at 80 K in $(\text{Sr,Ca})_3\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ induced by apical oxygen doping

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RECENT work^{1,2} has shown that superconducting copper oxyhalides can be synthesized, with the La_2CuO_4 structure but with no oxygen in the 'apical' positions outside the CuO_2 planes. For these materials to be rendered superconducting, charge carriers must be introduced into the CuO_2 planes; in previous work, positive carriers (holes) have been introduced by the incorporation of interstitial fluorine in $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ (transition temperature $T_c = 46$ K)¹, or by the substitution of sodium for calcium in $(\text{Ca,Na})_2\text{CuO}_2\text{Cl}_2$ ($T_c = 26$ K)². Here we present an alternative doping approach for this copper oxyhalide family: holes are introduced by partially replacing the (monovalent) halogen that occupies the apical sites with (divalent) oxygen. We have obtained bulk superconductivity with T_c above 77 K in a new double-layer compound $(\text{Sr,Ca})_3\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$, which is isostructural with $(\text{La,Sr})_2\text{-CaCu}_2\text{O}_6$ (ref. 3), with (Sr,Ca) at the (La,Sr) or Ca sites, and Cl at the apical sites outside the CuO_2 planes.

We synthesized $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ compounds using a high-pressure, high-temperature technique, which has proven effective in searching for new homologous series of high- T_c superconducting copper oxides^{4–6}. The precursors Sr_2CuO_3 , SrCuO_2 and Ca_2CuO_3 were prepared by solid-state reaction between CuO and SrCO_3 or CaCO_3 (each 99.9% pure). Precursor $\text{Sr}_2\text{CuO}_2\text{Cl}_2$ (Cl source of the sample) was obtained by the reaction between CuO and fully dehydrated SrCl_2 (99% pure)

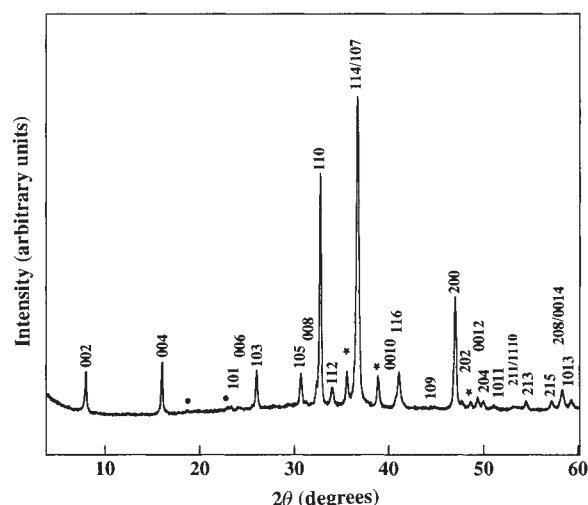


FIG. 1 X-ray powder diffraction pattern of $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$. Main peaks are assigned to a tetragonal structure (space group $I4/mmm$) with $a = 3.87$ Å and $c = 22.16$ Å. Also indicated are peaks from CuO impurity (*), and from unidentified minor phase(s) (●).

at 800 °C in flowing O_2 . The precursors were mixed, in an argon-filled glove box, with SrO_2 (99% pure) and CuO powders in a stoichiometry of $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ with 10% excess CuO. The materials were then subjected to high-pressure synthesis at 5.0 GPa at $\sim 1,000$ °C for 1 h using a cubic-anvil-type high-pressure apparatus. Adding excess CuO, which in most cases remained an impurity in the final products, to the starting materials was found to enhance the formation of the phase $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$. The role of SrO_2 is to create an oxygen atmosphere during the high-pressure synthesis, with the oxygen pressure controlled by the amount of SrO_2 in the nominal starting composition. In the absence of SrO_2 , the $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_4\text{Cl}_2$ product is an insulator.

By using a 0.4 molar ratio of SrO_2 in the nominal starting composition, we synthesized superconducting samples with the predominant phase being $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ for $0 \leq x \leq 1.0$ and $0.40 \leq y \leq 0.80$, as shown by the X-ray powder diffraction pattern in Fig. 1 for $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$. As expected, the

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sample also contained CuO and unidentified minor phase(s). We did not find any evidence from powder diffraction for other superconducting phases in these samples, such as $(\text{Sr,Ca})_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$ (refs 4, 7, 8), which can be formed at high pressure from the same cation-forming elements used here.

We determined the crystallography, microstructure and compositions of our samples using X-ray powder diffraction, high-resolution transmission electron microscopy (HRTEM) and energy dispersive X-ray spectroscopy (EDS) using a scanning electron microscope. $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ crystallizes in a tetragonal structure, with space group $I4/mmm$. It is isostructural with $(\text{La,Sr})_2\text{CaCu}_2\text{O}_6$, (ref. 3) with (La,Sr) or Ca replaced by (Sr,Ca), and with the apical oxygens replaced by Cl. Crystallographically, $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ constitutes the $n=2$ member of a new homologous series $(\text{Sr,Ca})_{n+1}\text{Cu}_n\text{O}_{2n}\text{Cl}_2$, with $\text{Sr}_2\text{CuO}_2\text{Cl}_2$ (refs 9, 10) being the $n=1$ member. As a result, in the ideal case of $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_4\text{Cl}_2$, all the apical oxygens of the CuO_2 planes are thus replaced by Cl. In our superconducting samples, however, EDS analysis (using $\text{Sr}_2\text{CuO}_2\text{Cl}_2$ as a reference material) reveals that the Cl content in the $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ grains is significantly less than 2. The average Sr:Ca:Cu:Cl atomic ratio over tens of well grown $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$ grains is approximately 34:14:32:20, in agreement with the suggested formula of the new compound. This result suggests that there is insufficient Cl to fully occupy the apical site. As the samples were synthesized under a high oxidizing pressure, it is reasonable to assume that a few oxygens are in turn incorporated in the apex site. The increase of c -axis length on going from $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_4\text{Cl}_2$ ($a = 3.89 \text{ \AA}$, $c = 22.02 \text{ \AA}$) to $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$ ($a = 3.87 \text{ \AA}$, $c = 22.16 \text{ \AA}$) can be explained by the expansion of the (Sr,Ca)-Cl rock-salt block due to enhanced Coulomb repulsion between the adjacent (monovalent) Cl layers resulting from the introduction of (divalent) oxygen. The reduction of the a dimension is expected for p-type carrier doping. Similar to the observation^{9,10} in $\text{Sr}_2\text{CuO}_2\text{Cl}_2$, the large Cl^- ion ($r(\text{Cl}^-) = 1.81 \text{ \AA}$)¹¹ leads to highly puckered (Sr,Ca)-Cl rock-salt layers as shown by the HRTEM image in Fig. 2A. Following the crystal structure model illustrated in Fig. 2B, we performed an X-ray Rietveld refinement for $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$. The chlorine occupancy at the apical site was considered as a variable, with the rest of this site being assumed to be substituted by oxygen. Table 1 summarizes the refinement results. The refinement gave a weighted-profile R -factor of 5.69%, indicating that the structural model is a reasonable one. The refined composition, $\text{Sr}_{2.3(5)}\text{Ca}_{0.7(5)}\text{Cu}_{2.0}\text{O}_{4.8(4)}\text{Cl}_{1.2(4)}$, is in reasonable agreement with the proposed formula for the new compound. However, because both EDS and X-ray Rietveld refinement are not sensitive to light elements such as oxygen in the crystal structure, the analysis performed here has large systematic error. These preliminary results should

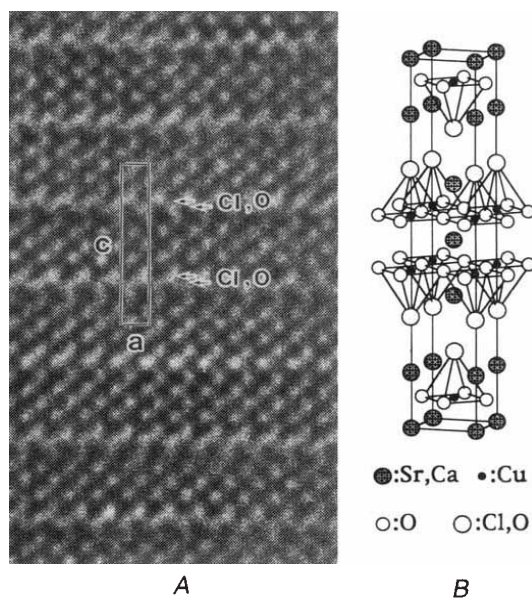


FIG. 2 A, Typical high-resolution transmission electron microscopy image taken along [100] in $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$ samples, revealing a long periodicity along the c direction. The excess oxygen was presumed to occupy the vacancy at the chlorine site. Due to the large ionic radius of Cl^- ($1.81 \text{ \AA}$)¹¹, (Cl, O) layers concomitantly shift away from the CuO_2 sheets, leading to a very puckered (Sr,Ca)-(Cl,O)-(Cl,O)-(Sr,Ca) rock-salt block. B, Schematic view of the crystal structure of $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$, which is isostructural with $(\text{La,Sr})_2\text{CaCu}_2\text{O}_6$, where (La,Sr) or Ca has been replaced by (Sr,Ca), and apical oxygens of the CuO_2 pyramidal planes have been replaced by Cl.

therefore be confirmed by other more precise measurements, such as neutron diffraction.

We characterized the superconducting properties of the samples by measuring the d.c. susceptibility using a SQUID magnetometer and the d.c. resistivity by the four-probe method. The superconducting samples typically have T_c s of 60–80 K and large Meissner volume fractions ($>20\%$ at 5 K). Figure 3 shows that $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$ undergoes a superconducting transition at 80 K. The Meissner volume fraction of 35% indicates bulk superconductivity. In reality, the sample is not single-phase, as revealed by the powder diffraction pattern. However, as no systematic correlation between the Meissner volume fractions and the amount of impurity phase(s) was found in the superconducting samples, it is reasonable to assign the superconducting transition to the $\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{2-y}$. This transition temperature is much higher than the 60-K T_c of $(\text{La,Sr})_2\text{CaCu}_2\text{O}_6$ (ref. 3), and is the highest T_c yet reported for a copper oxyhalide superconductor. The Fig. 3 inset shows the temperature dependence of the sample resistivity. The higher T_c^{onset} of $\sim 90 \text{ K}$ and the wider transition range in the resistivity data may imply that the sample is chemically inhomogeneous.

It is believed that a requirement for superconductivity in the layered copper oxides is the creation of charge carriers by either oxidizing (hole-doping) or reducing (electron-doping) the CuO_2 planes. The presence of the strong oxidizing atmosphere during our synthesis and the aforementioned lattice-parameter evolution suggest that these copper oxyhalide superconductors are hole-doped. The doping cannot be due to cation substitution² because Sr and Ca cations are isovalent, and is not likely to be due to interstitial anions¹ either, as we will discuss below. We suggest, instead, that the substitution of oxygen for (apical) Cl may provide a new hole-doping mechanism, 'apical oxygen doping'. Using the formula given by the Rietveld refinement, the charge balance resulting from the apical oxygen complement gives a hole concentration of up to 0.4 (2) per CuO_2 unit. The hole concentration for an optimized p-type high- T_c supercon-

TABLE 1 Crystallographic data for $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_2\text{O}_{4+\delta}\text{Cl}_{1.3}$

Atom	Site	x	y	z	Occupancy	$B_{\text{iso}} (\text{\AA}^2)$
(Sr/Ca) (1)	2a	0	0	0	0.5/0.5 (3)	3.0/1.0
(Sr/Ca) (2)	4e	0	0	0.1507 (8)	0.9/0.1 (2)	2.0/1.0
Cu	4e	0	0	0.428 (1)	1.0	1.0
O (1)	8g	0.5	0	0.428 (1)	1.0	3.0
O (2)	4e	0	0	0.31 (2)	0.38 (18)	2.0
Cl	4e	0	0	0.298 (8)	0.62 (18)	3.0

Space group, $I4/mmm$; unit-cell dimensions; $a = b = 3.8679 (2) \text{ \AA}$, $c = 22.161 (1) \text{ \AA}$; $R_w = 5.69\%$, $R_p = 4.22\%$, $R_e = 2.78\%$, $R_i = 4.49\%$, $R_f = 3.19\%$, where R_w , R_p , R_e , R_i and R_f are the R -factors for the weighted-profile, profile, expected, integrated intensity and structure factor of the fit, respectively. Cu $K\alpha$ radiation was used; the refinement range of 2θ was $25\text{--}110^\circ$. Values in parentheses represent the standard deviation in the last digit; B_{iso} is the isotropic thermal parameter; the total occupancy at each site of (Sr/Ca) (1), (Sr/Ca) (2) and Cl/O (2) was fixed as 1; the CuO_2 (1) was constrained to a plane.

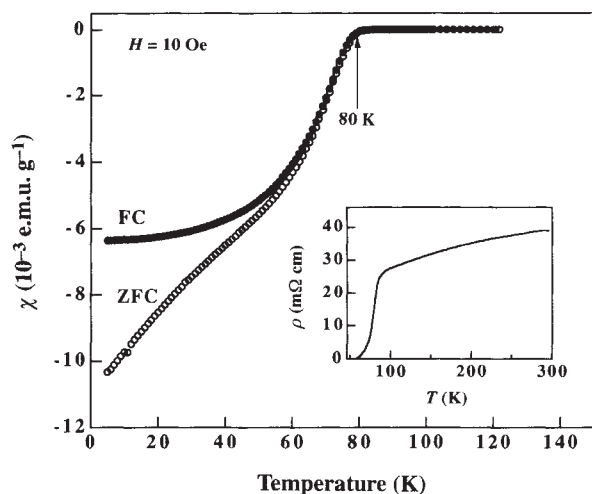


FIG. 3 Temperature dependence of d.c. magnetic susceptibility of $\text{Sr}_{2.3}\text{Ca}_{0.7}\text{Cu}_{2.0}\text{O}_{4+\delta}\text{Cl}_{1.3}$. The measurement was carried out using a d.c. SQUID magnetometer (Quantum Design) in both zero-field cooling (ZFC) and field cooling (FC) mode in a 10-Oe field. A bulk superconducting transition is observed at 80 K, with a Meissner volume fraction of 35%. Inset, resistivity as a function of temperature measured by the four-probe method with a 0.1-mA current.

ducting copper oxide usually¹² ranges from 0.12 to 0.25. If this 'rule' holds here, in principle apical oxygen doping is able to contribute sufficient carriers to support p-type superconductivity (perhaps a few vacancies at the apical site would lead to optimum doping).

In the Rietveld refinement we ignored the possibility of interstitial anions—a source of hole carriers known from other La_2CuO_4 -type materials^{1,13}. The most likely 'interstitial' site in this double-layer structure is the open 2b site in the Sr/Ca(1) spacer layer interleaved between the two adjacent pyramidal CuO_2 planes. The site (an apical position relative to the CuO_2 planes) is vacant in the ideal structure¹⁴. In view of the highly oxidizing condition and the ion size (1.40 Å for O^{2-} , and 1.81 Å for Cl^- (ref. 11), the probable interstitial anion here would be oxygen. But the incorporation of oxygen at this site in $(\text{La},\text{Sr})_2\text{CaCu}_2\text{O}_{6-\delta}$ (ref. 15) is known to suppress superconductivity suggesting that this 'interstitial' anion should not exist, or play a positive role in the present superconducting phase. Thus apical oxygen doping, a partial substitution of divalent oxygen for the monovalent apical halogen, appears the most plausible p-type carrier doping mechanism for this double-layer copper oxyhalide superconductor. This is the first time that p-type carrier doping has been achieved through anionic ion (not ion cluster¹⁶) substitution, analogous to the well established cation substitutions used in other high- T_c superconducting copper oxides.

There accordingly seems to be at least three carrier doping routes leading to p-type superconducting copper oxyhalides: interstitial halogen insertion¹, cation substitution² and anion substitution (this work). The significance of the present approach is that it has distinguished the effectiveness of apical oxygen as a dopant for oxidizing the CuO_2 plane for otherwise apical-oxygen-free materials. Apical oxygen doping (or its joint use with the other two mechanisms) may lead to further multi-layer high- T_c copper oxyhalide superconductors. □

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1. Al-Mamouri, M., Edwards, P. P., Greaves, C. & Slaski, M. *Nature* **369**, 382–384 (1994).
2. Hiroi, Z., Kobayashi, N. & Takano, M. *Nature* **371**, 139–141 (1994).
3. Cava, R. J. et al. *Nature* **345**, 602–604 (1990).
4. Adachi, S., Yamauchi, H., Tanaka, S. & Mōri, N. *Physica C* **208**, 226–230 (1993); **212**, 164–168 (1993).
5. Jin, C.-Q., Adachi, S., Wu, X.-J., Yamauchi, H. & Tanaka, S. *Physica C* **223**, 238–242 (1994); in *Advances in Superconductivity Vol. 7* (Springer, Tokyo, in the press).
6. Yamauchi, H., Tamura, T., Wu, X.-J., Adachi, S. & Tanaka, S. *Jap. J. appl. Phys.* **34**, L349–L351 (1995).

7. Hiroi, Z., Takano, M., Azuma, M. & Takeda, Y. *Nature* **364**, 315–317 (1993).
8. Laffez, P., Wu, X. J., Adachi, S., Yamauchi, H. & Mōri, N. *Physica C* **222**, 303–309 (1994).
9. Müller-Buschbaum, H. *Angew. Chem. int. Edn. engl.* **16**, 674–687 (1977).
10. Miller, L. L. et al. *Phys. Rev. B* **41**, 1921–1925 (1990).
11. Shannon, R. D. & Prewitt, C. T. *Acta Crystallogr.* **B25**, 925–945 (1969).
12. Zhang, H. & Sato, H. *Phys. Rev. Lett.* **70**, 1697–1699 (1993).
13. Chaillout, C. et al. *Physica C* **158**, 183–191 (1989).
14. Siegrist, T., Zahurak, S. M., Murphy, D. W. & Roth, R. S. *Nature* **334**, 231–232 (1988).
15. Shaked, H. et al. *Phys. Rev. B* **48**, 12941–12950 (1993).
16. Uehara, M., Nakata, H. & Akimitsu, J. *Physica C* **216**, 453–457 (1993).

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Synthetic molecules that fold into a pleated secondary structure in solution

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THE construction of synthetic molecules that fold or assemble predictably into large, well defined structures represents a fertile area of chemistry. Many supramolecular systems have been reported that self-assemble as a result of non-covalent interactions^{1–7}; and the control of higher-order protein structure by *de novo* design has also been demonstrated^{8,9}. Protein secondary structural motifs have also been stabilized by incorporating artificial groups that impose constraints on the folded architecture^{10–12}. Here we describe the synthesis of molecules that will fold in water into a pleated structure, as a result of interactions between alternating electron-rich donor groups and electron-deficient acceptor groups. We verify the pleated structure using absorption and NMR spectroscopy. Donor–acceptor interactions have been used previously to engineer specific supramolecular geometries^{2,13}, and are energetically favourable in organic as well as in aqueous solutions. But whereas previously such interactions have been used to effect self-assembly of distinct molecules, our results show that they can also determine the secondary structure of complex synthetic molecules in solution.

Our system incorporates 1,5-dialkoxynaphthalene and 1,4,5,8-naphthalenetetracarboxylic diimide (Fig. 1a) as aromatic donor and acceptor units, respectively. These moieties were chosen because they are readily functionalizable, and their donor–acceptor complex is relatively stable in aqueous solution. For example, monomers **1** and **2** have an association constant of 130 M^{-1} in water, as determined by standard NMR¹⁴ and ultraviolet-visible¹⁵ spectroscopic analysis.

Our aim was to assemble the donor and acceptor groups on a suitable backbone such that they would stack to give a pleated structure (Fig. 1b). To assist in design of an appropriate backbone, we obtained an X-ray structure of the co-crystal between the monomers **3** and **4** (Fig. 2). Taking this alignment of the rings as a probable energy minimum in solution, molecular mechanics simulations predicted that the backbone shown in Fig. 1c would be compatible with the stacked structure of the co-crystal, folding to give the secondary structure shown in Fig. 1d. In theory, any α -amino acid could be placed between the 1,5-dialkoxynaphthalene and 1,4,5,8-naphthalenetetracarboxylic diimide groups. Initially, we have chosen L-aspartic acid residues for the backbone so as to increase water solubility and prevent aggregation. The series **5–7** (Fig. 1c) was synthesized by solid-phase methods, and below we report spectroscopic evidence consistent with the

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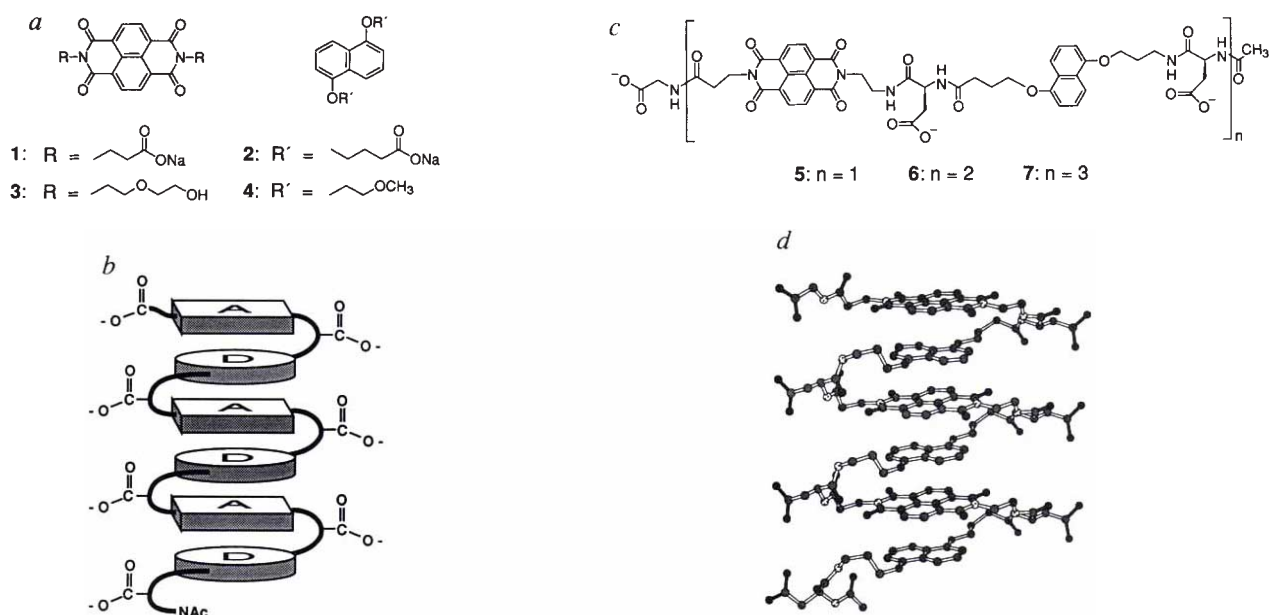


FIG. 1 **a**, Monomers used for aqueous binding titrations (**1** and **2**) and in the co-crystal (**3** and **4**) shown in Fig. 2. **b**, General schematic representation of a secondary structural element based on donor-acceptor interactions. **c**, Molecular structure of **5**–**7**. These molecules were synthesized by standard solid-phase methods using a strategy based on the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group¹⁹ with glycine-functionalized *o*-chlorotriyl polystyrene resin²⁰. Compounds **5**–**7** were purified using preparative reversed-phase liquid chromatography resulting in samples that were homogeneous by high-performance liquid chromatography. 500 MHz ¹H NMR (20 mM sodium phosphate buffer

at pH 7 in D₂O) spectra are entirely consistent with the above structures. Fast atom bombardment (FAB) high-resolution mass spectra: calculated *m/e* for C₄₈H₅₀N₇O₁₇ (**5** as free acid, M⁺ + H), 996.3263 and for C₉₂H₉₂N₁₃O₃₁ (**6** as free acid, M⁺ + H), 1,874.6022. Found, 996.3281 and 1,874.6077, respectively. FAB low-resolution mass spectrum: calculated *m/e* for C₁₃₆H₁₃₄N₁₉O₄₅ (**7** as free acid, M⁺ + H), 2,754.7. Found, 2,754. Detailed experimental procedures for the synthesis will be published elsewhere. **d**, Computer-generated representation of **7** based on X-ray structure of the **3**–**4** co-crystal (see Fig. 2) and calculated structure of the backbone.

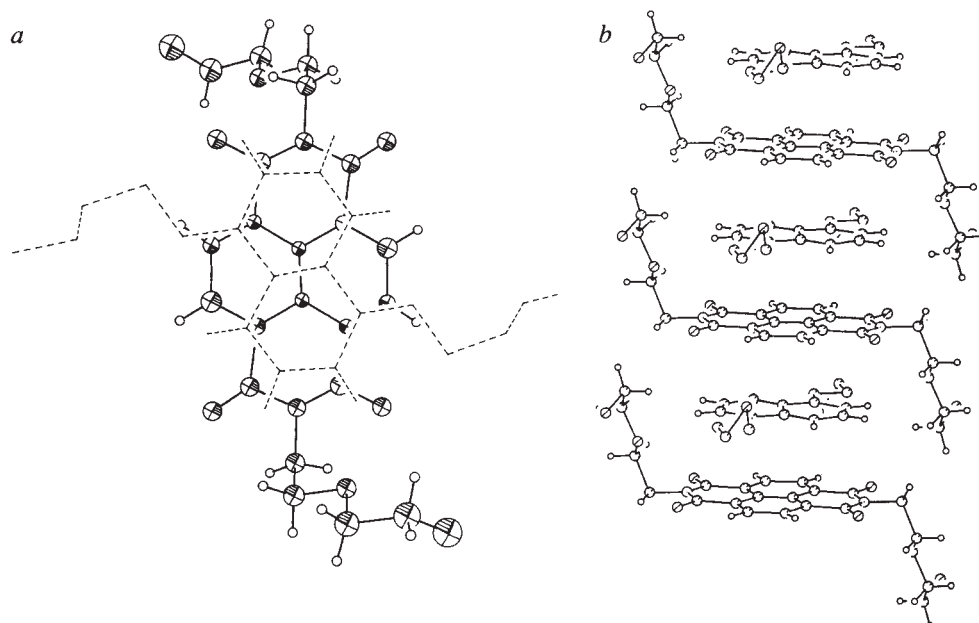
folding of **6** and **7** into a pleated structure in which the aromatic rings are stacked in a parallel, alternating fashion.

Aqueous solutions of **5**, **6** and **7** have a plum colour due to broad charge-transfer (CT) absorption bands in the visible region. These bands show linear Beer's law behaviour below 2 mM, indicating that observations made at or below these concentrations reflect only intramolecular interactions. As expected the wavelength of maximum absorption (λ_{max}) for the CT band of the dimer **5** (522 nm) is identical to that of the complex between monomers **1** and **2**. On the other hand, a red shift of

18 nm is observed for the tetramer **6** and hexamer **7**. Such a shift is consistent with our model for **6** and **7** in which more than two aromatic groups are stacked simultaneously¹⁶.

Significant hypochromism and shape differences are observed in the ultraviolet absorption bands of **5**–**7** when their ultraviolet-visible spectra are taken at identical chromophore concentrations (Fig. 3a). Assuming negligible effects due to the attached backbone, oligomers consisting of totally non-interacting chromophores would give rise to spectra equal in shape and intensity to that of a 1:1 mixture of monomers at the same concentration

FIG. 2 X-ray structure of the **3**–**4** co-crystal **a**, View down the *b* axis showing the π -stacking of the two molecules **3** and **4**. Only the major component of the disordered side-chain is shown. **4** is shown as dashed lines, while **3** is shown as thermal ellipsoids drawn to the 30% probability level. The molecules each lie around crystallographic inversion centres separated by a 1/2 *b*-axis translation (3.46 Å). **b**, View showing a portion of the extended π -stacking arrangement of **3** and **4** along the *b* axis. Atoms are represented as spheres of an arbitrary size. The solvent molecules have been omitted for clarity.



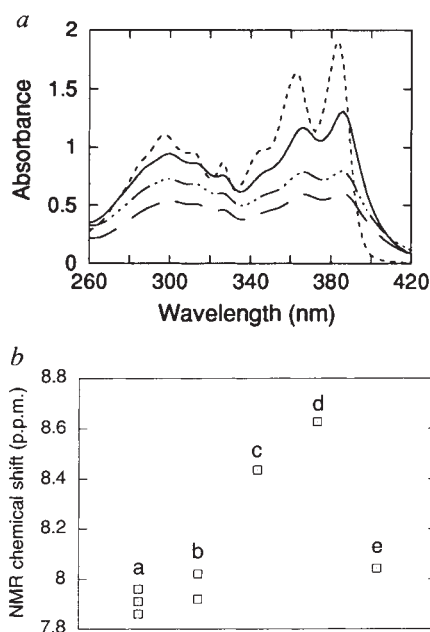


FIG. 3 *a*, Ultraviolet spectra of aqueous solutions (20 mM sodium phosphate at pH 7.0) of 1:1 ratio of **1** and **2** (----); **5** (—); **6** (— · —); **7** (—); at 7.5×10^{-5} M in each chromophore. *b*, Summary of average chemical shifts (taken as the midpoints of each AA'BB'q pattern) of aromatic diimide hydrogens of compounds **7** (a); **6** (b); **5** (c); **1** (d); **1** in 1:1 complex with **2** as extrapolated from binding titration (e). NMR spectra taken in 20 mM sodium phosphate/D₂O at pH (uncorrected) 7.0.

(dashed line in Fig. 3a). Hypochromism is dependent on distance r as a function of $1/r^3$ and highly orientation-dependent with respect to the angle formed by the ring planes¹⁷. Thus the increasing hypochromism and shape differences that are observed in the series **5–7** indicate that the aromatic chromophores are electronically coupled via a stacked arrangement in which the aromatic ring planes are parallel.

NMR data¹⁸ are all fully consistent with the folding of the molecules **6** and **7** into well defined pleated structures. The chemical shift of the diimide hydrogens of uncomplexed **1** can be taken as an approximation for that of a fully “unfolded” terminal diimide moiety, whereas the chemical shift of the fully complexed **1**, as extrapolated from the NMR binding titration of **1** with **2**, can be taken as that of a “folded” terminal diimide moiety (Fig. 3b). The average chemical shift for the diimide hydrogens of dimer **5** lies between these two “endpoints”, indicating that under the conditions of the NMR experiment the dimer is only partially folded. On the other hand, the farthest downfield (least complexed) diimide signals in both **6** and **7** lie slightly upfield from those of fully complexed **1**. Thus the carboxy-terminal acceptor groups are largely, if not entirely, stacked in **6** and **7**, and/or their electronic environments are influenced by stacking of the internal aromatic groups. In addition, two-dimensional COSY NMR spectra reveal that several of the methylene hydrogens on the backbones are diastereotopic, indicating a restriction of rotational motion on the NMR time-scale. Finally, the expected nuclear Overhauser effect enhancements are observed between hydrogens of adjacent aromatic rings (spectra are available; see Supplementary Information).

Taken together, the above spectroscopic evidence supports the conclusion that molecules **6** and **7** exhibit a well defined pleated structure despite the inherent flexibility of the backbone. This, in turn, confirms the postulate that donor–acceptor interactions can serve as the basis for stabilizing novel higher-order structural motifs.

We have named these pleated molecules aedamers, in reference to the aromatic electron donor–acceptor interactions that dictate

their folding arrangement. They can be prepared by an easy, modular synthetic route. As such, they define a new, and possibly general approach to the construction of large synthetic macromolecules with well defined higher-order structure. For example, the electronic structures of the chromophores could be manipulated to produce large arrays with novel optical properties. Alternatively, by changing the amino acids in the backbone, aedamers could be produced that are soluble in organic solvents, insert into membranes, interact with biological macromolecules, or assemble into even larger bundle-type structures. □

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1. Lindsey, J. S. *New J. Chem.* **15**, 153–180 (1991).
2. Amabilino, D. B. et al. *J. Am. chem. Soc.* **117**, 1271–1294 (1995).
3. Whitesides, G. M., Mathias, J. P. & Seto, C. T. *Science* **254**, 1312–1319 (1991).
4. Lehn, J.-M. *Angew. Chem. int. Edn engl.* **29**, 1304–1319 (1990).
5. Chambron, J. C., Dietrich-Buchecker, C. O., Heitz, V., Nierengarten, J.-F. & Sauvage, J.-P. in *Transition Metals in Supramolecular Chemistry* (eds Fabrizzi, L. & Poggi, A.) 371–390 (Kluwer Academic, Dordrecht, 1994).
6. Ashton, P. R., Philp, D., Spencer, J. & Stoddart, J. F. *J. chem. Soc., chem. Commun.* 1677–1679 (1991).
7. LaBrenz, S. R. & Kelly, J. W. *J. Am. chem. Soc.* **117**, 1655–1656 (1995).
8. Betz, S. F., Raleigh, D. P. & Degrad, W. F. *Curr. Opin. struct. Biol.* **3**, 601–610 (1993).
9. Quinn, T. P., Tweedy, N. B., Richardson, J. S., Williams, R. W. & Richardson, D. C. *Proc. natn. Acad. Sci. U.S.A.* **91**, 8747–8751 (1994).
10. Mutter, M. et al. *J. Am. chem. Soc.* **114**, 1463–1470 (1992).
11. Ghadiri, M. R., Soares, C. & Choi, C. *J. Am. chem. Soc.* **114**, 825–831 (1992).
12. Åkerfeldt, K. S., Kim, R. M., Comac, D., Groves, J. T., Lear, J. D. & DeGrado, W. F. *J. Am. chem. Soc.* **114**, 9656–9657 (1992).
13. Hunter, C. A. & Sanders, J. R. *M. J. Am. chem. Soc.* **112**, 5525–5534 (1990).
14. Hanna, M. W. & Ashbaugh, A. L. *J. phys. Chem.* **68**, 811–816 (1964).
15. Deranleau, D. A. *J. Am. chem. Soc.* **91**, 4044–4054 (1969).
16. Benniston, A. C., Harriman, A. & Lynch, V. M. *Tetrahedron Lett.* **35**, 1473–1476 (1994).
17. Cantor, R. C. & Schimmel, P. R. *Biophysical Chemistry Part II* 390–408 (Freeman, New York, 1980).
18. Chudek, J. A., Foster, R. & Twisleton, D. R. *J. chem. Soc., Perkin Trans. II* 1385–1389 (1983).
19. Barlos, K. et al. *Tetrahedron Lett.* **30**, 3943–3946 (1989).
20. Atherton, E. & Shepard, R. C. *Solid Phase Peptide Synthesis* (IRL, Oxford, 1989).

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Correlation between climate events in the North Atlantic and China during the last glaciation

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EPISODES of massive iceberg release (Heinrich events)^{1–3} into the North Atlantic Ocean during the last glaciation were associated with recurring episodes of unusually cold North Atlantic surface water (Bond cycles)⁴ and cold air temperatures over Greenland (Dansgaard-Oeschger events)^{5,6}. Four of the youngest of these cold events have also been reported in climate records from sites outside the North Atlantic region⁷, but until now the entire suite has been identified only in North Atlantic marine sediments, Greenland ice-core records and, tentatively, in French lake sediments⁸. Here we examine grain-size data from Chinese loess and intercalated accretionary palaeosols of last-glacial age for evidence of similar climate signals remote from the North Atlantic region. We see grain-size maxima with ages that match those of the last six Heinrich events, which we interpret as an indication of the

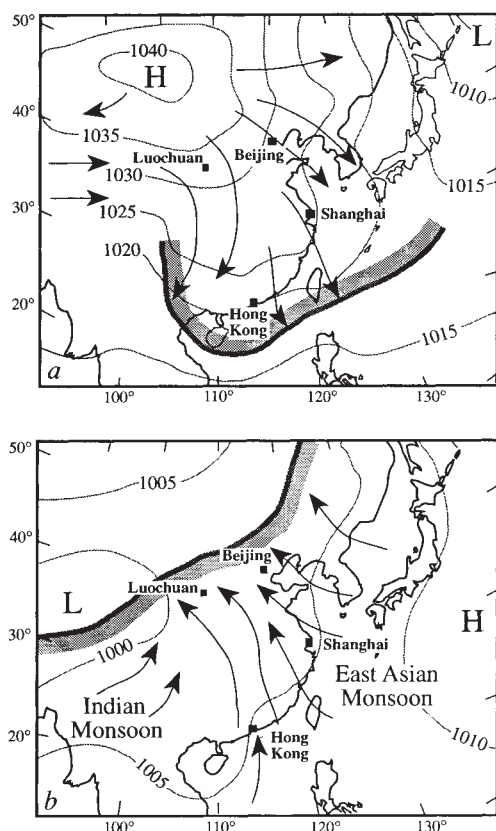


FIG. 1 Winter and summer monsoon regimes affecting China^{26–28}. *a*, January surface pressure (mbar), dominant wind vectors (arrows) and average southern limit of polar front (bold line). Northwestern winds associated with the Mongolian High and westerly winds from central Asia entrain the bulk of the aeolian dust delivered to the Loess Plateau. *b*, July surface pressure, dominant wind vectors and average landward limit of monsoon front. Summer monsoon precipitation is delivered to the Loess Plateau by the onshore flow of western Pacific maritime air which dominates the East Asian summer monsoon regime.

changing strength of the East Asian winter monsoon, which largely controls the transport and deposition of central Asian aeolian dust. Thus it seems that these Heinrich events have left their signature in the Chinese loess record. This is consistent with simulations of the glacial climate⁹, which imply that the climates of the North Atlantic and China were linked by the effect of westerly winds.

Chinese climate is characterized by seasonal alternations of the East Asian summer and winter monsoons (Fig. 1), for which the loess–palaeosol record constitutes an excellent long-term proxy record¹⁰. Precipitation-driven pedogenesis is largely responsible for the production of ultrafine-grained magnetic minerals that strongly influence the strength of the magnetic-susceptibility signal in the loess sequence¹¹. Variations in magnetic susceptibility correlate closely with the global marine $\delta^{18}\text{O}$ record, and have been used as an index of changing summer monsoon strength which varies with orbitally forced solar radiation¹². Modern dust storms and dust-fall events in China are associated with strong northwesterly winds, occur mainly in winter and spring, and historically are associated with dry, cold intervals¹³. The coarse fraction of the dust is greatest when the wind is strongest. On this basis, grain-size peaks in the loess record are inferred to record times when the winter monsoon strengthened, thereby increasing the competency of the dust-transporting winds¹⁴. The regional variation in grain size and thickness of Malan loess (last glaciation) implies deposition by a northwesterly wind system¹⁵, a direction matching that of present average winter monsoon winds¹⁰.

Magnetic susceptibility and grain size were measured through the upper ~12 m of two exposed sections near Luochuan

(35° 45' N, 109° 25' E) in the central Loess Plateau north of Xi'an, one at Potou¹⁵ and one nearby at Xiaheimu (Fig. 2). These sections are part of a continuous stratigraphic sequence forming the Luochuan yuan (loess platform). In this area, the S1, L1SS1 and S0 palaeosols (see Fig. 2 for nomenclature) are exposed in various gully walls at the same altitudes, and the shapes of magnetic-susceptibility profiles are essentially identical.

The coarse-grained fraction of atmospheric dust has been shown to correlate with the dry deposition flux¹⁶. In the Potou profile, we employ the median diameter of the chemically isolated quartz fraction, QMD, which we consider a more consistently reliable measure of dust flux than bulk grain-size measures because it excludes post-depositional pedogenic clay¹⁵. For comparison, in the Xiaheimu profile we analysed the total coarse silt (>40 μm) fraction, a more conventional proxy for dust flux, assuming mainly dry depositional conditions^{14,17}.

Because chronology is a central issue in this investigation, we have taken three approaches to dating the aeolian record. First, stratigraphically consistent thermoluminescence ages have been obtained from the Malan loess section at Luochuan¹⁸ (Fig. 2), but because 1σ errors range from 9 to 18%, considerable uncertainty is involved in using the dates for establishing a detailed (± 2 kyr) chronology. Second, we have correlated the magnetic susceptibility profile with the SPECMAP marine oxygen-isotope (MIS) time series and inferred that the rapid change in susceptibility values at the L1/S0 and S1/L1 stratigraphic boundaries correlate with the MIS 2/1 (12.05 kyr) and 5/4 (73.91 kyr) transitions, respectively¹⁹. We then derived a chronology by linear interpolation. Third, because the dust flux rate during the last glacial age varied with changing climate²⁰, we have used a grain-size model to calculate the age of successive stratigraphic levels between the MIS 2/1 and 5/4 transitions. The model assumes that coarse-grain-size maxima in the loess–palaeosol succession mark times when the competency and capacity of dust-bearing winds increased. We therefore further assume that the dust flux is proportional to grain size, and express the age, T , of a level (m) as:

$$T_m = T_1 + (T_2 - T_1) \left(\sum_{i=1}^m A_i^{-1} \right) \left(\sum_{i=1}^n A_i^{-1} \right)^{-1}$$

where T_1 and T_2 are the ages of the MIS 2/1 and 5/4 boundaries, respectively; A_i is the accumulation rate at any level (i), assumed to be proportional to either the QMD or >40- μm size fraction; and n is the number of stratigraphic levels between T_1 and T_2 . Calculated errors for this method range from ± 1 –3 kyr. We

TABLE 1 Ages of grain-size maxima for Potou QMD data derived by alternative dating methods

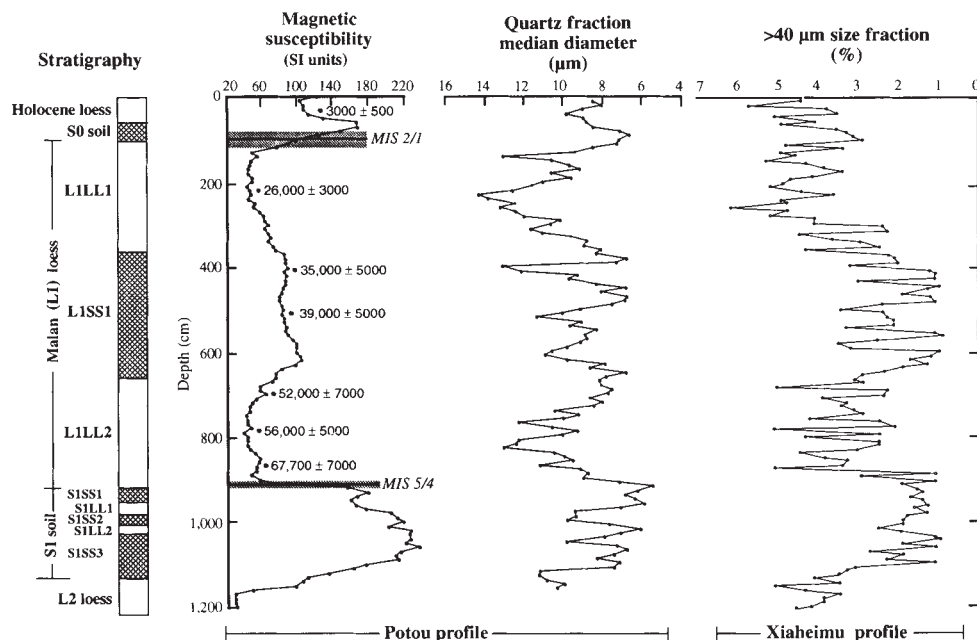
Stratigraphic depth of grain-size maximum (cm)	TL* (kyr)	Linear interpolation† (kyr)	Grain-size model‡ (kyr)
140	16.3	13.5	15.2
230	26.0	22.7	21.3
310	30.8	27.4	26.1
400	36.0	32.7	33.4
520	42.6	39.3	43.4
610	47.8	44.5	50.3
740	55.5	52.2	61.6
770	62.7	59.2	63.7
830	67.8	64.5	67.6
870	76.2	72.5	70.4

* Linear interpolation between ages derived from thermoluminescence (TL) measurements.

† Linear interpolation between MIS 5/4 and 2/1 transitions (approximately the top of palaeosols S1 and S0, respectively).

‡ Grain-size model applied between MIS 5/4 and 2/1 "stratigraphic windows" (Fig. 2).

FIG. 2 Variations with depth of loess and palaeosol properties at Luochuan. Stratigraphic units¹² were sampled at 10-cm intervals (supplemented in the Xiaheimu profile at 5-cm intervals from 110–160 and 910–970 cm depth). Thermoluminescence ages¹⁸ for the Potou profile are plotted with the magnetic-susceptibility data. Stratigraphic "windows" used in calculating ages for the Malan loess are based on marine isotope stage (MIS) boundaries 2/1 (12.05 kyr) and 5/4 (73.91 kyr)¹⁹. The exact positions of these MIS boundaries with respect to the susceptibility profiles is uncertain because of pedogenic effects; thus, for each we designated a stratigraphic "window" within which the stage boundary is inferred to lie (Fig. 2). The upper and lower limits of these windows (± 20 cm for MIS 2-1 and ± 10 cm for MIS 5/4) were used to calculate an average age and maximum and minimum error limits for each stratigraphic level. Quartz data are from Xiao *et al.*¹⁵.



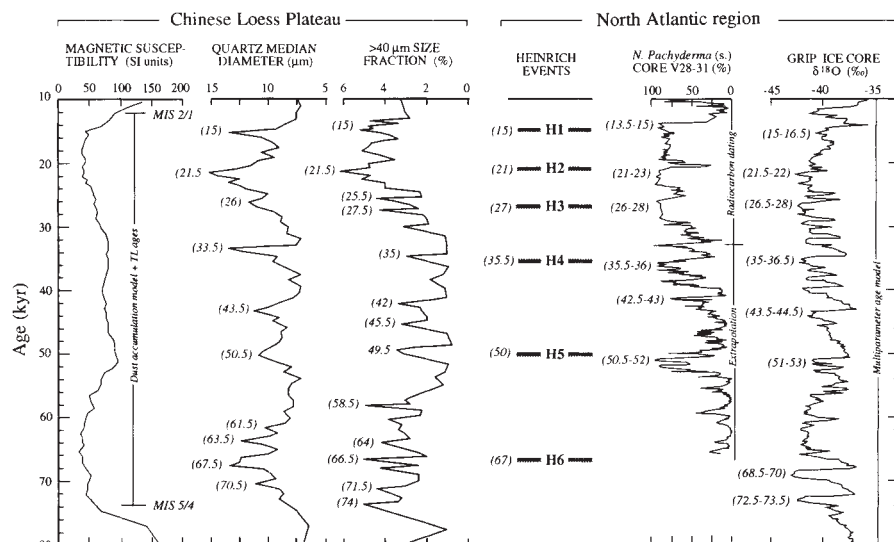
believe these ages to be more reliable than those obtained by the first two methods, because observed dust flux variability—rather than an assumed linear interpolation—is used to construct the chronology. At most horizons the differences among methods are < 5 kyr (Table 1).

The grain-size time series from Luochuan display a high-frequency, high-amplitude pattern, with the most prominent peaks culminating at approximately 15, 21.5, 25.5–27.5, 33.5–35, 42–43.5, 49.5–50.5, 63.5–64 and 66.5–67.5 kyr (Fig. 3). Both the pattern and timing of these grain-size variations bear a striking resemblance to palaeoclimate proxy records from the North Atlantic region that span the last glacial cycle (Fig. 3). In particular, peaks in the two grain-size time series match major changes in *Neogloboquadrina pachyderma* (s.) percentage in North Atlantic core V28–31⁴ (Bond cycles) and in $\delta^{18}\text{O}$ of the GRIP (Greenland) ice core⁵ (Dansgaard-Oeschger events). Lay-

ers of ice-rafted detritus (IRD) within the cold marine intervals record massive iceberg-discharge (Heinrich) events from the ice sheets around the North Atlantic at times of extreme atmospheric cooling, and date to approximately 15, 21, 27, 35.5, 50 and 67 kyr (refs 1–3), identical, within error limits, to the ages derived for the Chinese grain-size peaks. Nevertheless, the early parts of the marine, ice-core and loess records are not closely dated (Fig. 3), and so the inferred correlations of older events in the time series remain tentative.

Cold North Atlantic sea surface temperatures are known to have had a dramatic effect on regions directly downwind in Europe during the last glaciation and the late-glacial Younger Dryas interval^{21,22}. We believe that the Chinese loess deposits, still farther downwind, may record the same high-frequency climate events seen in the North Atlantic marine and ice-core records. Comparable high-frequency events have not been detected in

FIG. 3 Time series comparing magnetic susceptibility and grain-size variations in the Luochuan loess/palaeosol succession with North Atlantic Heinrich events^{1–3}, *Neogloboquadrina pachyderma* (s.) variations⁴, and Greenland ice sheet $\delta^{18}\text{O}$ variations⁵ during the last glaciation (80–10 kyr). Loess time series are dated by a sedimentation model keyed to stratigraphic "windows" representing the MIS 5/4 and 2-1 transitions. Ages of Heinrich events H1–H3 are based on accelerator mass spectrometry (AMS) dates bracketing IRD layers^{2,3}; the age of H4 is based on one AMS age⁴, whereas ages of H5 and H6 were obtained by extrapolation^{3,4}. The upper part of the *N. pachyderma* (s.) record is controlled by AMS ages, but ages of the lower part are based on extrapolation of ^{14}C -based sedimentation rates². The chronology of the upper 8.6 kyr of the GRIP core is controlled by annual layer counting and volcanic-acid reference horizons, whereas the lower part is based on a multiparameter method²⁹; estimated error limits range from 0.05 kyr at 8.6 kyr to 2 kyr at 40 kyr. Ages of peaks (in parentheses) are rounded to the nearest 0.5 kyr in all time series.



the East Asian summer monsoon proxy record, which primarily reflects a pedogenic response to the onshore flow of Pacific air. Thus these climate variations are apparently associated with the winter monsoon regime, and may be linked to the North Atlantic via the westerlies. High dust influx to Greenland ice during Dansgaard-Oeschger events^{23,24} may record a component of these same Chinese aeolian events. Climate-model simulations of full-glacial conditions are consistent with such an inference, for they point to enhanced atmospheric circulation and to latitudinal displacement of surface storm tracks; these tracks passed to the south of the North American ice sheets and then across the North Atlantic IRD zone, southern Europe, and central Asia towards the Loess Plateau^{9,25}. If this mechanism for a trans-continental teleconnection is correct, then an important component of Chinese palaeoclimate may be linked to changes in North Atlantic oceanic conditions. □

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1. Heinrich, H. *Quat. Res.* **29**, 142–152 (1988).
2. Bond, G. et al. *Nature* **360**, 245–249 (1992).
3. Grousset, F. E. et al. *Paleoceanography* **8**, 175–192 (1993).
4. Bond, G. et al. *Nature* **365**, 143–147 (1993).
5. Dansgaard, W. et al. *Nature* **364**, 218–220 (1993).

6. Grootes, P. M., Stuiver, M., White, J. W. C., Johnsen, S. & Jouzel, J. *Nature* **366**, 552–554 (1993).
7. Broecker, W. S. *Nature* **372**, 421–424 (1994).
8. Thouveny, N. et al. *Nature* **371**, 503–506 (1994).
9. Kutzbach, J. E., Guetter, P. J., Behling, P. J. & Selin, R. in *Global Climates Since the Last Glacial Maximum* (eds Wright, H. E. Jr et al.) 24–93 (Univ. Minnesota Press, Minneapolis, 1993).
10. An, Z. S. et al. *Quat. Int.* **7/8**, 91–95 (1990).
11. Verosub, K. L., Fine, P., Singer, M. & TenPas, J. *Geology* **21**, 1011–1014 (1993).
12. An, Z. S., Kukla, G. J., Porter, S. C. & Xiao, J. L. *Quat. Res.* **36**, 29–36 (1991).
13. Liu, T. S. et al. *Loess and the Environment* (China Ocean Press, Beijing, 1985).
14. Thurston, G. D. & Spengler, J. D. *Atmos. Envir.* **19**, 9–25 (1985).
15. Xiao, J., Porter, S. C., An, Z. S., Kumai, J. & Yoshikawa, S. *Quat. Res.* **43**, 22–29 (1995).
16. Slinn, W. G. N. in *Air-Sea Exchange of Gases and Particles* (eds Liss, P. S. & Slinn, W. G. N.) 299–396 (Reidel, Hingham, MA, 1983).
17. Zhang, X. Y. et al. *Quat. Res.* **41**, 35–43 (1994).
18. Forman, S. L. *Quat. Res.* **36**, 19–28 (1991).
19. Martinson, D. G. et al. *Quat. Res.* **27**, 1–29 (1987).
20. Beer, J. et al. *Geophys. Res. Lett.* **20**, 57–60 (1993).
21. Guiot, J., Pons, A., de Beaulieu, J. L. & Reille, M. *Nature* **338**, 309–313 (1989).
22. Rind, D., Peteet, D., McIntyre, A. & Ruddiman, W. *Clim. Dyn.* **1**, 3–33 (1986).
23. Taylor, K. C. et al. *Nature* **361**, 432–436 (1993).
24. Mayewski, P. A. et al. *Nature* **263**, 1747–1751 (1994).
25. Manabe, S. & Stouffer, R. J. *J. Clim.* **1**, 841–866 (1988).
26. Zhang, J. C. & Lin, Z. G. *Climate of China* (Wiley, New York, 1992).
27. Gao, Y. X. et al. *Some Problems on East Asia Monsoon* (Science Press, Beijing, 1962).
28. Jiao, B. C. et al. (eds) *Atlas of Chinese Physical Geography* (Map Press, Beijing, 1984).
29. Johnsen, S. J. et al. *Nature* **359**, 311–313 (1992).

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Compositions of near-solidus peridotite melts from experiments and thermodynamic calculations

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MID-ocean-ridge basalts (MORBs) are probably a mixture of liquids formed by near-fractional melting of upwelling mantle over a range of pressures^{1–3}. Thus, an understanding of MORB genesis requires knowledge of the compositions of near-solidus melts of mantle peridotite, which have not been measured experimentally. Here we present the results of melting experiments on peridotite using a two-stage diamond-aggregate extraction technique, and the results of thermodynamic calculations of peridotite melting. Both the experiments and the calculations show that at 10 kbar, near-solidus melts (melt fraction $F = 0.02$ – 0.05) of fertile peridotite are enriched in SiO_2 , Al_2O_3 and Na_2O and depleted in FeO^* (all iron as FeO), MgO and CaO relative to higher-degree melts. At $F \approx 0.02$, the partial melt has ~ 57 wt% SiO_2 and is qualitatively similar to silica-rich melt inclusions found in spinel peridotites worldwide^{4,5}. At low melt fractions ($F \leq 0.08$), measured and calculated clinopyroxene/liquid (cpx/liq) partition coefficients for TiO_2 are larger than those calculated from cpx–liq pairs in higher-melt-fraction experiments. This change in titanium partitioning just above the fertile peridotite solidus implies that other highly charged elements (such as Hf, Zr, Th and U) exhibit similarly complex behaviour during the initial stages of mantle melting.

The peridotite composition used in our experiments (Table 1) approximates 'primitive' upper mantle⁶ and was made from cleaned mineral separates (olivine (ol), orthopyroxene (opx),

cpx and spinel (sp); all $\leq 13 \mu\text{m}$ in size). Design of the single-stage and two-stage diamond-aggregate experiments and experimental tests for the attainment of equilibrium are described in detail in Baker and Stolper⁶. In a two-stage experiment, the peridotite sample is enclosed in graphite and platinum capsules, and is run for 96–174 h at controlled pressure (10 kbar) and temperature. After quenching, the peridotite plug is extracted from the capsule, reloaded with a layer of diamonds in a new capsule and rerun at the same pressure and temperature for 26–95 h. During the first stage, all of the liquid is in intimate contact with the residual peridotite and equilibrates with the residual minerals. Thus, the liquid that segregates into the diamond layer at the beginning of the second stage represents a near-equilibrium melt. Experimental conditions of the two-stage near-solidus runs are given in Table 1.

In Fig. 1 we plot selected oxide concentrations of the experimental glasses quenched from the diamond layer against temperature—all of the quenched liquids coexist with ol, opx, cpx and sp. Although all phases have been analysed, only glass compositions are reported in Table 1. The experiments at 1,250 °C are only 10 °C above the estimated solidus for this bulk composition⁶. With decreasing temperature, Cr_2O_3 , FeO^* , MgO and CaO decrease monotonically, although the slopes of the trends for the last three oxides steepen dramatically below $\sim 1,270$ °C. Between 1,330 and 1,270 °C, the partial melts have approximately constant silica contents (50–51 wt% SiO_2). Below 1,270 °C, the silica content of the melt increases dramatically, reaching 56.8 wt% at 1,250 °C (average of 69a and 70a liquids). The Na_2O content of the experimental liquid also increases dramatically below 1,270 °C, climbing to 5.7 wt% at 1,250 °C. The Al_2O_3 content increases linearly with decreasing temperature; at 1,250 °C the average Al_2O_3 content is 19.6 wt%. Between 1,330 and 1,280 °C, the TiO_2 concentration of the liquid increases with decreasing temperature, but below 1,280 °C it decreases. Between 1,300 and 1,250 °C, the Mg number ($\text{Mg\#}$, defined as molar $\text{Mg}/(\text{Mg} + \text{Fe}^*)$) of the melt decreases from 0.768 to 0.697 even though ol compositions are nearly constant ($\text{Mg\#}^{\text{ol}} = 0.909$ – 0.903). Calculated Fe^* –Mg exchange coefficients for ol–liq ($K_D = (\text{Fe\#}^{\text{ol}}\text{Mg\#}^{\text{liq}})/(\text{Fe\#}^{\text{liq}}\text{Mg\#}^{\text{ol}})$, where $\text{Fe\#} = (1 - \text{Mg\#})$) also decrease over this temperature interval (from 0.32 to 0.25). High-pressure experiments on silica- and alkali-rich melts have produced similarly low ol–liq K_D s (D. Draper, personal communication). Thermodynamic calculations (see

TABLE 1 Experimental conditions, glass compositions and bulk composition

Run no.	Press. (kbar)	Temp. (°C)	Time (h)	Silicate (mg)	Silicate† total	Crystalline‡ assemblage	SiO ₂	TiO ₂	Al ₂ O ₃	Cr ₂ O ₃	FeO*	MgO	CaO	Na ₂ O
62a	10	1,330	96/26	6.26	0.84	sp-lherz	49.7 (1)	0.47 (6)	15.4 (2)	0.26 (10)	6.7 (2)	12.8 (2)	12.7 (2)	1.74 (8)
73a	10	1,260	137/95	6.81	0.94	sp-lherz	53.1 (4)	0.62 (6)	18.6 (2)	0.08 (6)	5.3 (3)	8.5 (2)	9.3 (2)	4.1 (3)
69a	10	1,250	121/49	6.19	0.94	sp-lherz	56.4 (2)	0.55 (6)	19.8 (2)	0.02 (2)	4.2 (3)	5.4 (2)	6.6 (4)	5.8 (5)
70a	10	1,250	174/46	6.92	0.95	sp-lherz	57.3 (2)	0.59 (6)	19.3 (2)	0.04 (4)	4.2 (1)	5.4 (1)	6.7 (1)	5.7 (3)
85	10	1,240	115	7.33		sp-lherz								
87	2	1,150	166	14.7		sp-lherz + plag								
88§	10	1,200	106	9.4		sp-lherz + tr plag								
BCR-1							55.7 (3)	2.34 (8)	14.1 (1)		12.3 (3)	3.3 (1)	7.1 (1)	3.1 (2)
MM3¶							45.5	0.11	3.98	0.68	7.18	38.3	3.57	0.31

All experiments were two-stage runs and all of the charges contained ol, opx, cpx and sp, in addition to glass. Multiple run times indicate the number of hours of the first stage (no diamonds in the capsule) and second-stage (peridotite + diamonds). Individual diamonds were 44–54 μm in diameter. Glasses were analysed with a Camscan scanning electron microscope (SEM) fitted with an energy dispersive system; beam current was 0.15 nA measured in a Faraday cup in the sample holder and spectra were collected for 200 s. The raw counts were reduced using CITZAF³² after first normalizing the unknown *k*-ratios to those of a basaltic glass standard (VG-2³³) that was analysed during each SEM session. Each average glass analysis (wt%) represents the mean of 5–7 spot analyses; values in parentheses are 1σ , for example, 49.7 (1) = 49.7 ± 0.1 .

† Mass ratio of silicate powder (MM3) to total mass of material (MM3 + diamond) in each second-stage run.

‡ Crystalline phases present in each charge, sp-lherz indicates ol + opx + cpx + sp, tr indicates trace.

§ Ground run product from 87 was the starting material for run 88. Dramatic decrease in the modal abundance of plagioclase between runs 87 and 88 determined by X-ray diffraction and reflected-light microscopy. Back-scattered electron images show that the remaining trace plagioclase grains are not in contact with olivine; we infer that plagioclase is not stable under the conditions of run 88.

|| Mean and 1σ of 50 analyses of fused BCR-1 (10 kbar, 1,500 °C, 0.5 h). Analyses of this secondary glass standard were collected over a period of several months.

¶ MM3, bulk composition used in this study, made of crushed minerals ($\leq 13 \mu\text{m}$) separated from a Kibbourn Hole spinel lherzolite nodule.

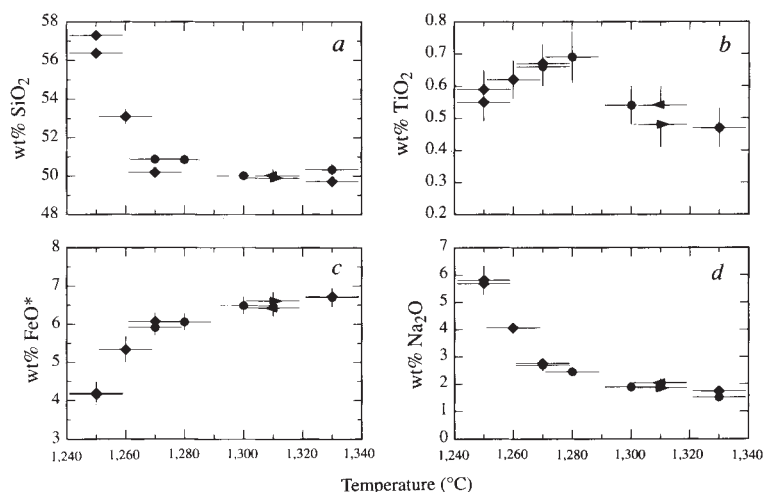
below) also predict a qualitatively similar decrease in ol-liq Fe*–Mg K_D near the solidus.

Several lines of evidence suggest that the unusual liquid compositions produced in the lowest-temperature runs (for example, the high silica and alumina contents) are not experimental artefacts. (1) Run durations for the two-stage experiments reported here are >2–3 times longer than required⁶ to reverse liquid compositions at 1,310 °C. Moreover, the 170–232 h run times were sufficient to produce unzoned olivines and spinels and nearly homogeneous pyroxenes in the residual peridotite layers. (2) Oxide totals for the glasses are 100 ± 1 wt%, and thus the high silica contents of the glasses are not related to high dissolved water contents. (3) Although some small cpx grains (<5 μm) are present in the diamond layer in the 1,250 °C runs, their compositions and equant morphologies indicate that they are not quench products (compositions of cpx from the diamond and peridotite layers overlap at the 2σ level). If present, quench overgrowths on these cpx grains could not have significantly modified the liquids in the diamond layers, because glass compositions in both 1,250 °C runs are similar and relatively homogeneous (Table 1). In contrast, the compositions of quenched glasses within the peridotite layer are extremely heterogeneous (for example⁶, 5–11 wt% MgO). (4) As discussed below, independent thermodynamic calculations of peridotite melting predict the same liquid compositional trends as observed in the experiments. (5) Forward and reversal experiments indicate that

plagioclase is not a stable subsolidus phase at 10 kbar in the MM3 bulk composition (Table 1); thus the high silica and alumina contents of the low-temperature glasses are not artefacts of a failure to nucleate plagioclase (plag). Published experiments on natural peridotite compositions^{7–14} show that bulk composition (especially Cr₂O₃ and Na₂O contents) exerts a fundamental control on the location of the plag + sp-lherzolite to sp-lherzolite transition in *P*–*T* space; increasing the Cr₂O₃ content at constant Na₂O content results in a decrease in the pressure of the plag + sp-lherzolite to sp-lherzolite transition. The relatively high Cr₂O₃ and moderate Na₂O contents of MM3 place this bulk composition within the sp-lherzolite field at 10 kbar based on published experiments, confirming our reversal experiments on this composition.

Thermodynamic calculations of isobaric partial melting of peridotite have been performed using MELTS, an algorithm that calculates crystal–liquid equilibria in silicate systems by thermodynamic potential minimization^{15–17}. Because none of the diamond-aggregate experiments presented here, in Baker and Stolper⁶ or in Hirose and Kushiro¹¹ were used to calibrate MELTS, the calculations are independent predictions of peridotite melting trends. The thermodynamic calculations at 10 kbar show the same compositional trends as the experiments. In particular, they show the same dramatic increases in SiO₂ content and decreases in TiO₂ content seen in the experimental results at $F < 0.07$ (Fig. 2a, b). Although SiO₂ contents of all of the

FIG. 1 Run temperature plotted against selected oxide concentrations in interstitial glasses from diamond layers of the single-stage runs (filled circles⁶), the two-stage runs (filled diamond symbols; Table 1, 1,330, 1,260 and 1,250 °C, and Baker and Stolper⁶, 1,270 °C) and the temperature-reversal experiments (arrow-heads⁶). For the reversals, the direction that the arrow-head points indicates whether the final run temperature (1,310 °C) was approached from the high (left-pointing) or low temperature (right-pointing) side. Error bars for oxide concentrations are 1σ ; if not shown, error bars are smaller than the symbols. Uncertainties in temperature are all taken as ± 9 °C.



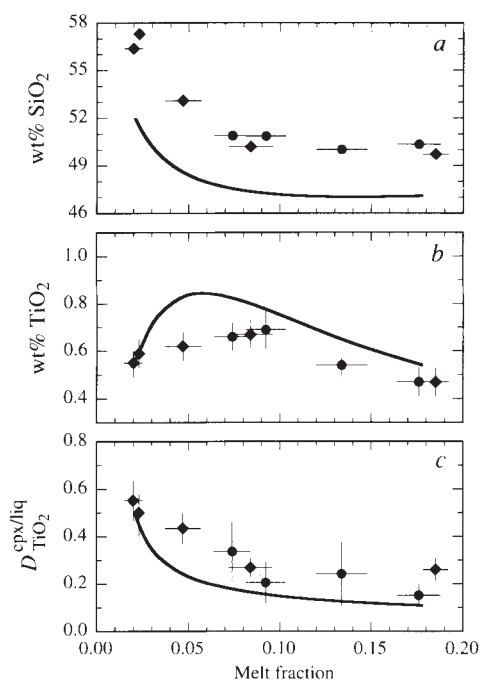


FIG. 2 a, Wt% SiO₂ in experimental and calculated liquids plotted against melt fraction. Symbols for single-stage and two-stage runs as in Fig. 1; melt fractions calculated by mass balance using the compositions of all phases. Solid line indicates liquids predicted by thermodynamic calculations on a bulk composition identical to that used in the experiments. Thermodynamic calculations were performed at 10 kbar and for oxygen fugacities equivalent to one order of magnitude below quartz–fayalite–magnetite using MELTS, which is calibrated using experimentally determined crystal–liquid equilibria^{16,17}. Although 1-atm experiments provide the bulk of the phase equilibrium constraints for the MELTS calibration, experimentally measured volumes, thermal expansivities and compressibilities allow extrapolation to higher pressures^{34,35}. b, TiO₂ contents (wt%) of liquids derived from small degrees of partial melting of fertile spinel peridotite at 10 kbar. With decreasing *F*, TiO₂ contents in the experimental melts go through a maximum at *F* ≈ 0.09, whereas the maximum occurs at *F* = 0.06 for the calculated liquids. Below *F* = 0.06, TiO₂ contents in the calculated liquids decrease more rapidly than in the experimental melts. c, Experimentally determined and calculated partition coefficients (wt-based) of TiO₂ between cpx and liquid, $D_{Ti}^{cpx/liq}$. The dramatic increase in $D_{Ti}^{cpx/liq}$ seen at low melt fraction in both the experiments and the calculations is attributed primarily to changes in cpx composition.

predicted melts are lower than those of the experiments owing to inaccuracies in the thermodynamic models for the ferromagnesian silicates¹⁷, the shapes of the trends are nearly identical.

The increase in the SiO₂ content of the liquid at low *F* is caused by the increase in Na₂O concentration (resulting from its incompatibility), which is known to reduce the activity coefficient of silica in the melt^{18,19}; that is, because the coexistence of ol + opx in peridotite essentially fixes the activity of SiO₂, any decline in the activity coefficient of silica in the liquid must be compensated by an increase in its silica content. Similarly, the expansion of the olivine phase volume accompanying¹⁸ the enrichment in Na₂O results in a dramatic decrease in MgO and FeO* contents of partial melts with decreasing *F* (Fig. 1c). The high sodium content of liquids near the solidus is also probably ultimately responsible for the observed drop in the ol–liq Fe*–Mg *K_D* at low *F*, which is also reproduced by MELTS calculations.

The anomalous decrease in the TiO₂ content of partial melt with decreasing *F* in both the experiments and the calculations (Fig. 2b) is caused by two factors. First, cpx, the mineral with the largest partition coefficient for TiO₂ comprises a greater proportion of the mode at low *F*. More importantly, $D_{Ti}^{cpx/liq}$

(TiO₂^{cpx}/TiO₂^{liq}, by weight) increases by a factor of 2.5–3.5 below *F* = 0.08 in both experiments and calculations (Fig. 2c). We attribute the marked change in partitioning of TiO₂ to changes in both liquid and solid compositions, but the primary cause is probably changes in pyroxene composition during early stages of melting. TiO₂ substitutes into cpx via charge-coupled substitutions²⁰, and thus the TiO₂ content of pyroxene is strongly dependent on the availability of charge-coupling cations (for example, Na⁺, Fe³⁺ and Al³⁺). Because these elements behave incompatibly, they are most abundant in cpx at low *F*. For this reason, we speculate that partitioning of other highly charged cations that substitute into pyroxene by charge-coupled substitutions (such as the rare-earth elements, the high-field-strength elements, U and Th) may also show strong compositional dependencies during the earliest stages of peridotite melting. This hypothesis is supported by recent synthetic basalt experiments that show that $D_{U,Th,Zr}^{cpx/liq}$ of U, Th and Zr increase with the Na and Al content of cpx²¹. Fractional fusion will magnify the importance of changes in cpx–liq partition coefficients, because cpx compositions change more rapidly per melting increment during fractional fusion than during batch fusion³.

Our results have several implications for understanding mantle melting and observed compositional variations of basalts. Calculations indicate that low-melt-fraction liquids can be substantially more viscous than typical basalts or higher-degree melts of peridotite. For example, the liquid from run 70a (1,250 °C; *F* = 0.023), has a calculated²² anhydrous viscosity of 2,040 P, as compared to a calculated value of 45 P for the liquid from run 62a (1,330 °C; *F* = 0.18). The addition of moderate amounts of water to the lowest-melt-fraction liquids would only partly mitigate this difference; if there were 2.0 wt% H₂O in the liquid in run 70a, its calculated viscosity at 1,250 °C would be 760 P. The high viscosities calculated for the 2% melt could require a revision in current thinking about the ease with which near-solidus melts of fertile peridotite could segregate from their residual crystals. In particular, the viscosity of the 2% partial melt (Table 1) is 20–200 times greater than that used in recent calculations^{23–25}, which will raise²⁶ timescales for melt segregation by factors of 5–15. This implies that during initial melting of fertile peridotite, liquid may not escape from the residue as readily as currently believed, and thus porosities might reach a few per cent in upwelling mantle that has just crossed the solidus²⁵. In this case, fractional fusion would only begin to dominate after the earliest melts had been extracted. Note that this effect will not apply to melting of Na₂O-poor depleted peridotites or to very volatile-rich small-degree melts.

The effect of increased partition coefficients in near-solidus melts documented for TiO₂, and inferred for other highly charged cations, could be important for understanding trace element contents of magmas derived in whole or in part from low-degree partial melts. In particular, U and Th may be less efficiently extracted from a peridotitic residue and certain incompatible trace element ratios thought to be invariant during melting may, in fact, vary. For example, strong variation in $D_{Ti}^{cpx/liq}$ during peridotite melting calls into question the practice of using K₂O/TiO₂ ratios of basalts to characterize the compositional variation in basalt source regions^{27–30}, because these ratios will vary during the first 5–10% melting. Participation of varying amounts of low-melt-fraction liquids may therefore lead to local variation in incompatible element ratios, and may also cause such ratios to vary for regionally averaged compositions if the total extent of melting is low, as believed to be the case for the deeper parts of the mid-ocean ridge (for example, the Arctic ridge or the southwest Indian ridge²⁷).

Although these conclusions are based on experiments and calculations at 10 kbar, both preliminary experiments and thermodynamic calculations indicate that 20-kbar near-solidus melts exhibit similar compositional trends. Increases in SiO₂ in near-solidus melts may be less pronounced at pressures substantially greater than 10 kbar. Increases in pressure cause $D_{Ti}^{cpx/liq}$ to

increase³¹ (though $D_{\text{Na}}^{\text{solid/liq}}$ increases by smaller amounts) and cause overall liquid SiO_2 values to decrease¹¹. $D_{\text{Ti}}^{\text{cp}/\text{liq}}$ also increases in the lowest-melt-fraction liquids at 20 kbar.

Our 2% partial melt is qualitatively similar to siliceous melt inclusions from both sub-continental and sub-oceanic spinel peridotites^{4,5}. These inclusions contain 54–65 wt% SiO_2 , 16–23 wt% Al_2O_3 , ≤ 8 wt% $\text{FeO}^* + \text{MgO}$, < 10 wt% CaO and 4–13 wt% $\text{Na}_2\text{O} + \text{K}_2\text{O}$, and though volatiles probably play a role in their genesis, our results indicate that nominally anhydrous alkali-rich melts coexisting with a spinel lherzolite assemblage near 10 kbar will be similar in composition. At such pressures, when the ratio of melt to peridotite is small, any alkali-rich silicate liquid will react with spinel peridotite to produce a silica-rich melt. Preservation of siliceous glass inclusions may be fostered by high viscosities and low diffusivities. □

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- Ahern, J. L. & Turcotte, D. L. *Earth planet. Sci. Lett.* **45**, 115–122 (1979).
- McKenzie, D. P. *J. Petrology* **25**, 713–765 (1984).
- Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. *J. geophys. Res.* **95**, 2661–2678 (1990).
- Draper, D. S. *J. Geol.* **100**, 766–776 (1991).
- Schiano, P. & Clocchiatti, R. *Nature* **368**, 621–624 (1994).
- Baker, M. B. & Stolper, E. M. *Geochim. cosmochim. Acta* **58**, 2811–2827 (1994).
- Takahashi, E. & Kushiro, I. *Am. Miner.* **68**, 859–879 (1983).
- Bertka, C. M. & Holloway, J. R. *Contr. Miner. Petrol.* **115**, 313–322 (1994).
- Falloon, T. J. & Green, D. H. *Miner. Petrol.* **37**, 181–219 (1987).
- Fujii, T. & Scarfe, C. *Contr. Miner. Petrol.* **90**, 18–28 (1985).
- Hirose, K. & Kushiro, I. *Earth planet. Sci. Lett.* **114**, 477–489 (1993).
- Jakes, A. L. & Green, D. H. *Contr. Miner. Petrol.* **73**, 287–310 (1980).
- Sen, G. *Nature* **299**, 336–338 (1982).
- Takahashi, E. *J. geophys. Res.* **91**, 9367–9380 (1986).
- Ghiorso, M. S. *Geochim. cosmochim. Acta* **58**, 5489–5501 (1994).
- Ghiorso, M. S. & Sack, R. O. *Contr. Miner. Petrol.* (in the press).
- Hirschmann, M. M., Stolper, E. M. & Ghiorso, M. S. *Mineralog. Mag.* **58A**, 418–419 (1994).
- Kushiro, I. *Am. J. Sci.* **275**, 411–431 (1975).
- Ryerson, F. J. *Geochim. cosmochim. Acta* **49**, 637–649 (1985).
- Sack, R. O. & Ghiorso, M. S. *Contr. Miner. Petrol.* **118**, 271–296 (1994).
- Lundstrom, C. C. et al. *Earth planet. Sci. Lett.* **128**, 407–423 (1994).
- Shaw, H. R. *Am. J. Sci.* **272**, 870–893 (1972).
- Iwamori, H. *Earth planet. Sci. Lett.* **114**, 301–313 (1993).
- Spiegelman, M. & Elliott, T. *Earth planet. Sci. Lett.* **118**, 1–20 (1993).
- Tanimoto, T. & Stevenson, D. J. *J. geophys. Res.* **99**, 4549–4558 (1994).
- McKenzie, D. *Earth planet. Sci. Lett.* **74**, 81–91 (1985).
- Langmuir, C. H., Klein, E. M. & Plank, T. in *Mantle Flow and Melt Generation at Mid-ocean Ridges* (eds Morgan, J. P., Blackman, D. K. & Sinton, J. M.) 183–280 (Geophys. Monogr. No. 71, Am. Geophys. Un., Washington DC, 1992).
- Sinton, J. M., Smaglik, S. M., Manoharan, J. J. & Macdonald, K. C. *J. Geophys. Res.* **96**, 6133–6155 (1991).
- Schilling, J. -G. et al. *Am. J. Sci.* **283**, 510–586 (1983).
- Shen, Y. & Forsyth, D. W. *J. geophys. Res.* **100**, 2211–2236 (1995).
- Blundy, J. D., Falloon, T. F. & Wood, B. J. (abstr.) *Eos* **74**, 658 (1993).
- Armstrong, J. T. in *Microbeam Analysis—1988* (ed. Newbury, D. E.) 239–246 (San Francisco Press, San Francisco, 1988).
- Jarosewich, E., Nelen, J. A. & Norberg, J. A. *Smithson. Contr. Earth Sci.* **22**, 68–72 (1972).
- Kress, V. C. & Carmichael, I. S. E. *Contr. Miner. Petrol.* **108**, 82–92 (1991).
- Lange, R. A. & Carmichael, I. S. E. *Geochim. cosmochim. Acta* **51**, 2931–2946 (1987).

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Trade-offs between life-history traits and a secondary sexual character in male collared flycatchers

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It has often been suggested that sexual selection may have important consequences for life-history evolution and vice versa^{1–5}. We manipulated the parental effort of male collared flycatchers (*Ficedula albicollis*) by changing the number of offspring in their nests and found a trade-off between parental effort and the size of the male's forehead patch (a secondary sexual character) in the following year. We report here that, in addition to this intra-generational trade-off, we found an inter-generational trade-off: the size of the forehead patch in first-year males was negatively related to the change in brood size of the nest in which they were raised. This has consequences for reproductive success because males with large patches mate with more females and have higher lifetime reproductive success. To our knowledge, this is the first experimental demonstration that life-history traits and secondary sexual characters trade off against each other. Our results support the suggestion that the life-history consequences of sexual ornaments are important in their evolution^{2–4}.

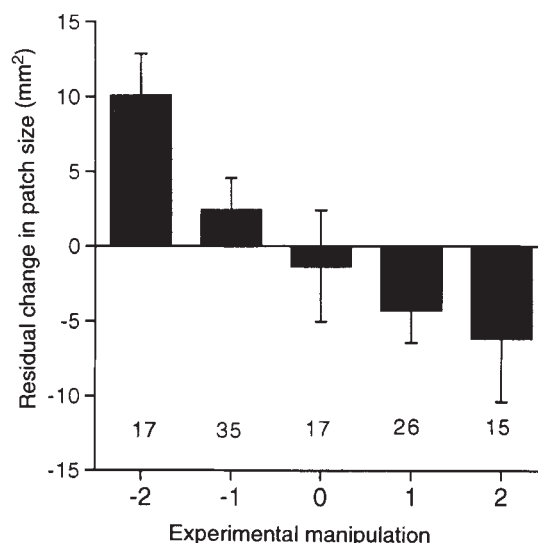
Current reproductive effort is expected to trade off against future reproductive output⁶. If secondary sexual characters are condition dependent and costly to their bearers, they can be considered a form of reproductive effort in the same way as can life-history traits^{7,8}. Consequently one can expect trade-offs between life-history traits and secondary sexual characters. A reduction in secondary sexual character size could therefore be expected after increased reproductive effort, which might have the effect of reducing reproductive success in subsequent years through lower sexual attractiveness or reduced competitive ability.

Collared flycatchers are small, migratory, sexually dichromatic passerines that breed in nest boxes and natural holes in trees. Males are black and white, with a conspicuous, variably sized white forehead patch (mean size index (height $\times$ width) = $81.5 \text{ mm}^2 \pm 16.34 \text{ s.d.}$, c.v. = 20.05, $n = 84$ adults older than 1 calendar year), which is absent or much smaller in females. The forehead patch is important in aggressive interactions between males: the outcome of experimentally staged disputes over nest box ownership can be predicted by the relative size of the patches, with males possessing larger patches being more likely to win a dispute (T. Pärt and A. Q., manuscript in preparation). In an analysis of all breeding attempts between 1981 and 1988 there is a significant positive relationship between a male's mean lifetime patch size and his mean recruitment of offspring per breeding attempt, controlling for lifespan and mating status (partial regression coefficient, $\beta = 0.004 \pm 0.001 \text{ s.e.}$, $F_{1,1171} = 6.61$, $P = 0.01$). Forehead patch size is determined during a partial moult in the African winter quarters preceding the spring migration⁹. We studied the effect of experimentally altered reproductive effort on the size of the forehead patch in subsequent years by performing brood-size manipulations on breeding pairs of flycatchers on the Swedish island of Gotland between 1983 and 1985 and in 1989 and 1990. Experimentally increased brood size has previously been shown to decrease future reproductive success in female flycatchers^{10,11} but there was no evidence of similar costs for males¹⁰.

We found (Fig. 1) a highly significant relationship between experimental treatment and the patch size of males in the year after the experiment. Males that had received an experimental increase in brood size had smaller forehead patches in the following year, whereas males that had been subjected to a decrease in brood size had larger patches in the following year. The difference in patch size change between the two extremes of experimental manipulation is large, amounting to more than one standard deviation in the size of the patch on unmanipulated males (Fig. 1).

Our results strongly suggest that male forehead patch size is a condition-dependent trait, sensitive to reproductive effort in the previous year. They therefore also provide the first evidence for a non-mortality cost of reproduction for males¹². We consider it unlikely that resources required to grow the trait limit its size, as might be the case for exaggerated sexual ornaments,

FIG. 1 Relationship between experimental treatment (number of nestlings added or removed) and relative change in forehead patch size ($X \pm \text{s.e.}$) for male collared flycatchers, data for 1983–85 and 1989–90 combined. The relationship is significant (ordered heterogeneity test²², $r_{spc} = 0.997$, $n = 110$, $k = 5$, $P < 0.0001$). Sample sizes (number of males recaptured and measured in year following experiment) are given below bars.



METHODS. One or two nestlings were moved at 1–2 days of age between nests paired by laying date and clutch size in 1983–85; in 1989–90 manipulations of brood size were done by exchanging complete clutches of eggs between nests with the same laying date: thus manipulations were not independent of original brood size. The forehead patch size of males was measured using callipers to the nearest 1.0 mm (by L.G.) when adults were feeding large nestlings. Nests were assigned into experimental groups randomly with respect to male characteristics: there were no significant differences across experimental groups in male patch size in the year of the experiment (ANOVA: $F_{4,105} = 2.03$, $P = 0.10$; regression: $F_{1,108} = 0.02$, $P = 0.89$). Male patch size was measured in the following year without knowledge of the experimental treatment to which males had been subjected in the previous year. As there was a significant negative relationship between original patch size and the difference between patch sizes in the two years across all experimental groups (regression, $F_{1,108} = 20.51$, $P < 0.0001$), we controlled for original patch size by calculating the residual from a regression of patch size change on original patch size. Patch size increases with age (A.Q., unpublished data) but there were no differences in the mean ages of males across experimental groups (Kruskal–Wallis $H_4 = 3.53$, $P = 0.47$). Although there were differences in experimental protocol between the two groups of years, we pooled data because the relationship between experimental treatment and patch size change was similar in both cases (ANCOVA: no differences between slopes ($F_{1,107} = 0.04$, $P = 0.8$) or elevations ($F_{1,107} = 0.08$, $P = 0.8$)). The

relationship was significant within both groups of years considered separately (ordered heterogeneity tests: 1983–85: $r_{spc} = 0.985$, $n = 75$, $k = 5$, $P < 0.0002$; 1989–90: $r_{spc} = 0.564$, $n = 35$, $k = 5$, $P < 0.04$). We used ordered heterogeneity tests²² because in each case the expected rank order of means across experimental groups was specified by the hypothesis tested. Our conclusions are not changed if linear regression is used.

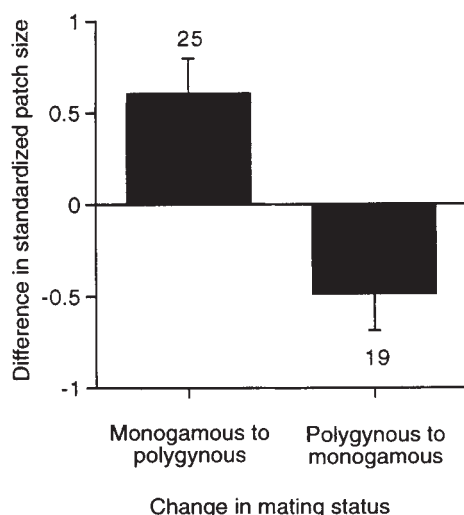


FIG. 2 Change in standardized forehead patch size ($X \pm \text{s.e.}$) for males that changed mating status (from monogamous to polygynous and vice versa) between successive years; numbers give sample sizes. Males that became polygynous also increased in forehead patch size (paired $t = 3.16$, 24 d.f., $P = 0.004$); males that became monogamous after being polygynous decreased in forehead patch size (paired $t = 2.41$, 18 d.f., $P = 0.027$). Combining these groups of males, an increase in the number of females a male was mated to (independent of order) is significantly associated with an increase in relative patch size (paired $t = 4.02$, 43 d.f., $P = 0.0002$).

METHODS. Males were classified as to mating status on the basis of the number of females' nests they were caught at while feeding young. Many polygynous males are misclassified as monogamous using this method¹⁵ because they feed the young of secondary females rarely, but because we consider only changes in mating status our test is conservative. Male patch size was standardized ($X = 0$; $\text{s.d.} = 1$) within age classes to control for age effects on patch size; each male appears only once and no male changed mating status more than once.

particularly because the patch is an unpigmented area. Rather, the forehead patch seems to be an example of a 'badge of status'¹³, for which the costs of possessing a large badge are socially enforced¹⁴. Although there was no evidence that males subject to an experimental increase in brood size had lower reproductive success in subsequent years, as measured by survival¹⁰, or laying date, clutch size and fledgling number (unpublished data), this does not mean that the observed changes in patch size did not affect other unmeasured components of male reproductive success such as the probability of becoming polygynous or obtaining extra-pair fertilizations. Both are relatively frequent in our study population^{15,16}, and other studies show that they may contribute substantially to variance in male reproductive success in birds^{17,18}. A relationship between forehead patch size and the probability of becoming polygynous was confirmed by an analysis of patch size changes in relation to changes in mating status for individual males between 1981 and 1988 (Fig. 2). Males that became polygynous after being monogamous had grown relatively bigger patches, over the winter; males that became monogamous after having been polygynous had grown relatively smaller patches (Fig. 2). Polygynous males recruit significantly more offspring to the breeding population than monogamous males¹⁵.

Our data further show that the size of the forehead patch developed by yearling males recruiting from nests in which brood size had been manipulated was related to the experimental manipulation (Fig. 3): males from increased brood-size nests developed smaller forehead patches. This result parallels the reduced fecundity of females recruiting from the same experimental nests^{10,19} and suggests that the causal mechanism for reduced patch size in these offspring is a reduction in condition of young from enlarged broods^{10,15,19}. Together, these results (Figs 1 and 3) indicate the existence of both inter- and intra-generational trade-offs between brood size and the size of the male forehead patch.

The fact that semelparous organisms tend to possess more exaggerated secondary sexual traits than iteroparous organisms suggests the existence of a trade-off between future reproduction

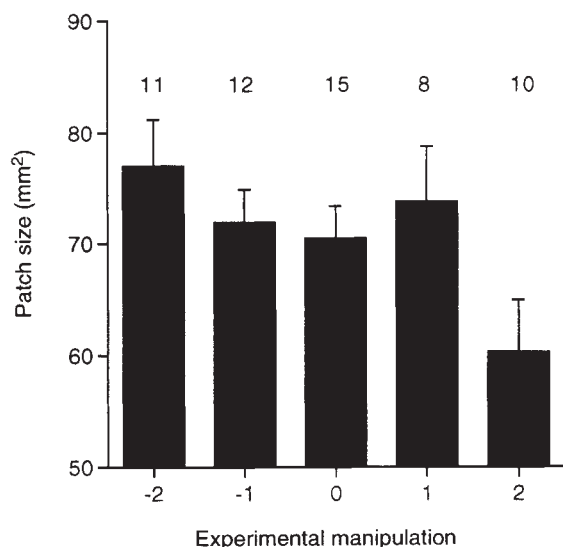


FIG. 3 Relationship between experimental manipulation (change in brood size) and forehead patch size ($\bar{X} \pm \text{s.e.}$) of first-year males recruited from experimental nests (mean per nest). The relationship is significant (ordered heterogeneity test²², $r_{SP} = 0.663$, $k = 5$, $P < 0.02$). Patch size in males was measured at the time when they were feeding nestlings in their first year. Numbers above bars show number of males. The analysis is restricted to experiments done in 1983–85 as brood-size manipulations were not independent of clutch size in 1989–90; there were no indications of year effects on patch size of recruited males (multiple regression with years as dummy variables, $P > 0.47$).

and ornamentation², as do some comparative studies of mortality in relation to sexual dimorphism²⁰, but this is the first direct evidence for a trade-off. The relationship between patch size and polygyny found in this study suggests that increased parental effort may reduce male attractiveness in subsequent years, by reducing the size of a condition-dependent secondary sexual character. Because increased parental effort by males may reduce sexually selected components of reproductive success, models of parental investment²¹ or sexual ornamentation⁸ must consider the trade-off between the two. □

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- Trivers, R. L. in *Sexual Selection and the Descent of Man* (ed. Campbell, B.) 136–179 (Aldine, Chicago, 1972).
- Partridge, L. & Endler, J. A. in *Sexual Selection: Testing the Alternatives* (eds Bradbury, J. W. & Andersson, M. B.) 265–277 (Wiley, Chichester, 1987).
- Andersson, M. *Sexual Selection* (Princeton Univ. Press, Princeton, 1994).
- Møller, A. P. *Sexual Selection and the Barn Swallow* (Oxford Univ. Press, Oxford, 1994).
- Höglund, J. *Etologia* **3**, 143–150 (1993).
- Williams, G. C. *Am. Nat.* **100**, 687–690 (1966).
- Andersson, M. *Biol. J. Linn. Soc.* **17**, 375–393 (1982).
- Nur, N. & Hasson, O. *J. theor. Biol.* **110**, 275–297 (1984).
- Cramp, S. & Perrins, C. M. (eds) *Handbook of the Birds of Europe the Middle East and North Africa Vol. 7* (Oxford Univ. Press, Oxford, 1993).
- Gustafsson, L. & Sutherland, W. J. *Nature* **335**, 813–815 (1988).
- Gustafsson, L. & Pärt, T. *Nature* **347**, 279–281 (1990).
- Lessells, C. M. in *Behavioural Ecology: An Evolutionary Approach* 3rd edn. (eds Krebs, J. R. & Davies, N. B.) 32–68 (Blackwell, Oxford, 1991).
- Hansen, A. J. & Rohwer, S. *Anim. Behav.* **34**, 69–76 (1986).
- Clutton-Brock, T. H. & Parker, G. A. *Nature* **373**, 209–216 (1995).
- Gustafsson, L. in *Lifetime Reproduction in Birds* (ed. Newton, I.) 75–88 (Academic, London, 1989).
- Gelter, H. P. et al. *Ibis* **134**, 62–68 (1992).
- Gibbs, H. L. et al. *Science* **250**, 1394–1397 (1990).
- Kempnaers, B. et al. *Nature* **357**, 494–496 (1992).
- Schluter, D. & Gustafsson, L. *Evolution* **47**, 658–667 (1993).
- Promislow, D. E. L. et al. *Proc. R. Soc. B* **250**, 143–150 (1992).
- Westneat, D. F. & Sherman, P. W. *Behav. Ecol.* **4**, 66–77 (1993).
- Rice, W. R. & Gaines, S. D. *Proc. natn. Acad. Sci. U.S.A.* **91**, 225–226 (1994).

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Hybrid speciation accompanied by genomic reorganization in wild sunflowers

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THE origin of a new diploid species via hybridization is theoretically difficult because it requires the development of reproductive isolation in sympatry. In the absence of isolation, the hybrid genotype will be overcome by gene flow with its parents. According to genetic models^{1–3}, reproductive isolation can be facilitated by rapid karyotypic evolution in the recombinant hybrid. Here we use comparative linkage mapping^{4,5} to demonstrate extensive genomic reorganization in the hybrid species *Helianthus anomalus*, relative to its parents *H. annuus* and *H. petiolaris*. The unprecedented detail provided by the linkage maps indicates that rapid karyotypic evolution in *H. anomalus* results from the merger of pre-existing structural differences between the parents, as well as chromosomal rearrangements apparently induced by recombination. Moreover, determination of the parental origin of mapped loci in *H. anomalus* suggests that parental genomic structure has influenced hybrid genomic composition by protecting several large linkage blocks from recombination during speciation. These mapping data, when combined with previous meiotic analyses⁶ and evidence of semisterility between the hybrid and its parents^{6,7}, satisfy genetic models for speciation through hybrid recombination.

The three species under investigation are self-incompatible annual sunflowers with the same chromosome number ($n = 17$)⁸. The parents, *Helianthus annuus* and *H. petiolaris*, are widespread polytypic species that are distinguished by several morphological and chromosomal features⁸, occur in divergent clades based on chloroplast DNA⁹ and nuclear ribosomal DNA¹⁰ variation, and have different ecological requirements. In general, *H. annuus* is restricted to heavy, clay soils and *H. petiolaris* to dry, sandy soils. Nevertheless, the two species are sympatric throughout much of western USA, and hybrid swarms are common. First-generation hybrids are semisterile, with pollen viabilities typically less than 10% and seed set less than 1% (refs 6 and 11), whereas F_2 pollen viability is highly variable, ranging from 13% to 97% (ref. 11).

By contrast, the hybrid *H. anomalus* is a rare species endemic to xeric habitats in northern Arizona and southern Utah^{7,12}, well within the range of its parents. Although *H. anomalus* is morphologically distinct from *H. annuus* and *H. petiolaris*, it combines their ribosomal DNA repeat units¹⁰ and allozymes⁹, as predicted for a diploid hybrid species¹³. In addition, individuals of the species possess the chloroplast DNA haplotype of *H. annuus* or *H. petiolaris* rather than a unique one⁹.

We generated genetic linkage maps for all three species by using random amplified polymorphic DNA (RAPD)¹⁴ markers (Fig. 1; see also Supplementary Information). To map dominant markers, intraspecific hybrids were generated between geographically isolated populations of each species and then crossed to an inbred sunflower line (CMS89). Mapping progenies of 50 to 60 individuals were grown to a size sufficient for total DNA isolations¹⁵, and selected individuals were surveyed for genetic polymorphisms shared by at least two mapping populations. From a survey of 715 single primers or primer pairs, 174 single primers and 13 primer pairs were chosen for mapping. For each mapping population, all segregating loci were scored, and linkage maps were generated with the computer program MAPMAKER (ref. 16). Loci were distributed onto 17 linkage groups in each map, corresponding to the haploid chromosome number

of the three species. Because of differences in levels of polymorphism among mapping populations, the number of loci mapped varied from 212 in *H. annuus* to 400 in *H. petiolaris* (Fig. 1; see also Supplementary Information). Total map length also varied by a factor of two (Fig. 1; also Supplementary Infor-

mation), but map density was similar among the three species, with an average distance between loci ranging from 4.6 cM in *H. petiolaris* to 6.2 cM in *H. anomalus*.

By comparing the genomic location and linear order of homologous markers, we inferred chromosomal structural rela-

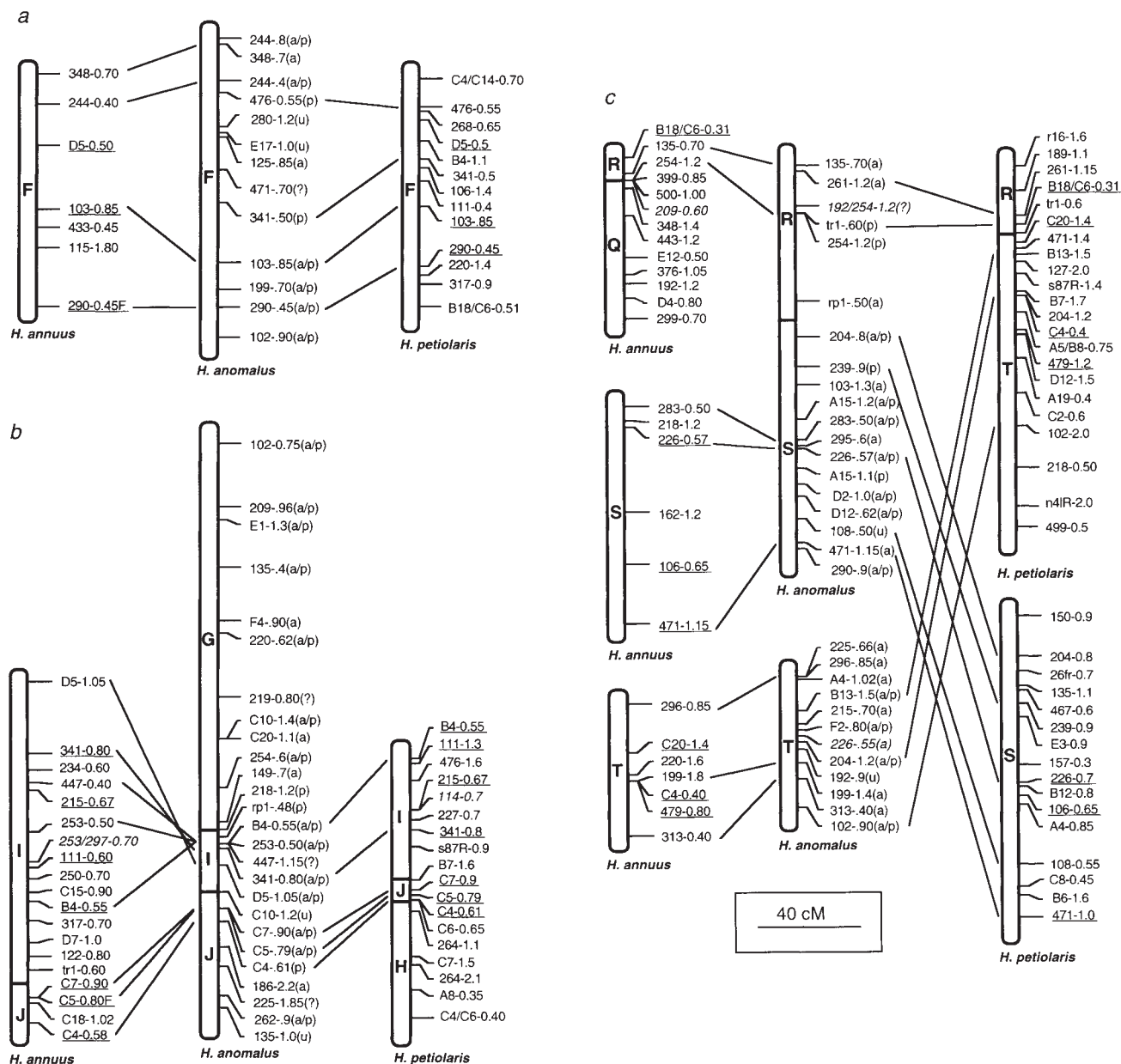


FIG. 1 Selected linkages from the two parental species, *H. annuus* and *H. petiolaris*, and their hybrid, *H. anomalus*, which illustrate the three types of structural relationships observed among the linkage maps: a, complete collinearity; b, inversions; and c, interchromosomal translocations. Letters within each linkage group designate major linkage blocks and indicate their relationship to homologous linkages in the other species. Locus nomenclature includes, from left to right, the primer designation and the size in kilobases of the segregating fragment scored. For the *H. anomalus* linkages, the letters in parentheses following each locus/allele indicate its parental origin: a, *H. annuus*; p, *H. petiolaris*; a/p, *H. annuus* or *H. petiolaris*; u, unique to *H. anomalus*; and ?, data not available. Lines between linkages indicate homologous loci between the hybrid species and its parents, whereas homologous loci between the two parental species are underlined. Loci italicized also show homology with loci from other mapping populations, but their genomic locations are incongruent with most homologous loci, suggesting paralogous rather than orthologous relationships.

METHODS. Primers 101–500 are from the University of British Columbia Biotechnology Laboratory, and primers A1–A20, B1–B20, C1–C20, D1–D20, E1–E20 and F1–F20 are from Operon Technologies. The remaining primers are 20–30 bases in length, and sequences can be obtained from the authors upon request. In some instances, RAPD primers were paired for amplification. Amplification and electrophoresis with single primers followed standard RAPD protocols¹⁴, whereas for amplification with RAPD primer pairs the extension time was reduced from 2 min to 1.0 s, and electrophoresis was performed on 2.0% agarose gels. Homology among RAPD fragments generated by the same primer was verified by Southern hybridization¹⁵ or by restricting gel-isolated fragments and observing congruent band profiles²¹. Maps were developed with the computer program MAPMAKER (ref. 16) using a Lod score of 4.0 and a recombination limit of 0.25. Recombination values were converted to map distances using the Kosambi mapping function. Potential scoring and ordering errors were detected using the 'genotype' command of MAPMAKER, and areas with apparent discrepancies were rescored or retested.

tionships among the three species (Figs 1 and 2; also Supplementary Information). Six linkages were collinear among all three species, whereas the remaining 11 linkages were not conserved in terms of gene order. The two parental species, *H. annuus* and *H. petiolaris*, differ by at least ten separate structural rearrangements, including three inversions and a minimum of seven interchromosomal translocations.

The genome of the hybrid, *H. anomalus*, was extensively rearranged relative to its parents (Figs 1 and 2; see also Supplementary Information). For 4 of the 11 rearranged linkages, *H. anomalus* shared the linkage arrangement of one parent or the other. For the remaining 7 linkages, however, unique linkage arrangements were displayed. In fact, a minimum of three chromosomal breakages, three fusions, and one duplication are required to achieve the *H. anomalus* genome from its parents. All seven novel rearrangements in *H. anomalus* involve linkage groups that are structurally divergent in the parental species, suggesting that structural differences may induce additional chromosomal rearrangements upon recombination¹⁷.

In addition to generating genomic maps for each species, we also attempted to determine the parental origin of each locus mapped in *H. anomalus*, by surveying natural populations of each parental species for loci or alleles mapped in *H. anomalus*¹⁵. A preliminary survey of 162 loci¹⁵ revealed that most *H. anomalus* linkage groups had loci derived from both parental species. However, several large blocks of markers appeared to have been transmitted intact from one or the other parental species, and it was speculated that these regions were protected from recombination by structural differences in the parental genomes¹⁵. Parental origin data from all 357 mapped loci (Fig. 1 and Supplementary Information) confirm the integrity of three of these blocks of genes, all of which occur in structurally divergent linkage groups (Figs 1 and 2; also Supplementary Information). All three blocks are derived from one of the parental species, *H. annuus*. A possible mechanism for the preservation of several linkage blocks from a single parent might be epistasis.

To determine whether the blocks of genes preserved in the hybrid are longer (in terms of an unbroken sequence of markers from one parent) than would be expected by chance, simulations of marker distributions within the *H. anomalus* genome were performed. Because markers that were polymorphic in both parents or unique to *H. anomalus* provide no information about recombination, the simulations were restricted to the 157 markers of known parental origin. Thus for each simulation we randomly sampled the length and distribution of blocks of genes within the entire genome, as well as within individual linkage groups or rearranged linkage blocks. The simulations were repeated 1,000 times. Results from the simulations revealed that three unbroken sequences of six, seven and seven or more markers from one parent, such as those observed in *H. anomalus*, would be unlikely to occur by chance alone ($P=0.021$).

To reduce gene flow, chromosomal structural differences must enhance reproductive isolation. This does appear to be the case in *H. anomalus*, where first-generation hybrids with their parents are partially sterile, with pollen stainabilities of 1.8% to 4.1% (*H. annuus*) and 2.0% to 58.4% (*H. petiolaris*)^{6,7}. Meiotic analyses revealed multivalent formations, and bridges and fragments, suggesting that chromosomal structural differences are largely responsible for hybrid semisterility⁶.

Because individual selection cannot explain the evolution of hybrid sterility¹⁸, the accumulation of additional chromosomal rearrangements in the ancestral hybrid population should be viewed as a chance consequence of recombination rather than an adaptive response to selection. Although these structural changes currently serve to reduce gene flow between *H. anomalus* and its parents, their role during the origin of the species is less clear. Theory suggests that the higher the number of structural polymorphisms in the ancestral hybrid population, the lower is the probability of generating new structural homozygotes². Outcrossing, which is typical of *Helianthus*⁸, would also make

fixation more difficult^{2,3}. Selfing can be induced in hybrids of *H. annuus* and *H. petiolaris*¹¹, but its frequency in natural hybrids is unknown. However, structural homozygotes generated from highly polymorphic hybrid populations are more likely to be unique and intersterile with each other and with their parents². Thus the presence of additional structural rearrangements in hybrid populations will reduce the probability of generating fertile hybrid progeny, but if fertile recombinants are produced they are less likely to be swamped out of existence by gene flow.

Genetic models for hybrid recombination¹⁻³ imply ecological divergence as well as karyotypic evolution, because the stabilized hybrid must either coexist with its parents or become adapted to a new habitat or niche³. This model is corroborated in *Helianthus*, as *H. anomalus* shows ecological divergence from both parents, occupying xeric habitats. A similar pattern of chromosomal and ecological divergence has been reported for two other diploid hybrid species in *Helianthus*^{10,19}.

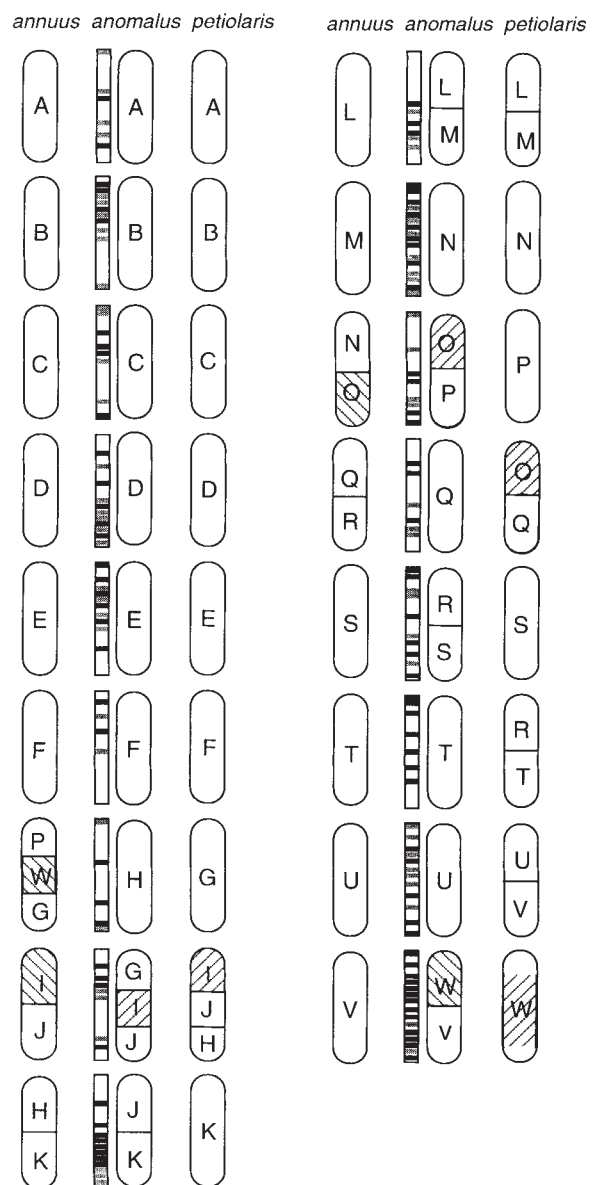


FIG. 2 Inferred structural relationships of chromosomes of the three *Helianthus* species, based on comparative linkage mapping. Lines of shading within chromosomes indicate inversions. Vertical rectangles adjacent to *H. anomalus* chromosomes indicate the distribution of markers from the two parental species: *H. annuus* and *H. petiolaris* markers are indicated by black and grey bars, respectively.

In addition to confirming theoretical genetic models for speciation by hybrid recombination, this study demonstrates the utility of comparative linkage mapping for studying the genomic processes accompanying or facilitating different modes of speciation. Not only can these maps be used to infer precisely the genomic structural changes accompanying speciation, but they also allow the genomic contribution of parental taxa to hybrid species or introgressant populations to be determined on a chromosome by chromosome basis, as well as within chromosomes. Future comparative mapping studies of species-level questions could be enhanced by the development of microsatellite loci²⁰, because most of these loci are likely to be polymorphic even in very narrow intraspecific mapping populations, and homology among loci can be assumed without additional experiments such as those required here (Fig. 1 and Supplementary Information). Microsatellite markers are currently being developed for *Helianthus* mapping populations. □

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1. Stebbins, G. L. *Cytologia* (Suppl. Vol.), 336–340 (1957).
2. Grant, V. *Cold Spring Harb. Symp. quant. Biol.* **23**, 337–363 (1958).
3. Templeton, A. R. *A. Rev. Ecol. Syst.* **12**, 23–48 (1981).
4. Bonierbale, M. W., Plaisted, R. L. & Tanksley, S. D. *Genetics* **120**, 1095–1103 (1988).
5. Tanksley, S. D., Bernatsky, R., Lapitan, N. L. & Prince, J. P. *Proc. natn. Acad. Sci., U.S.A.* **85**, 6419–6423 (1988).
6. Chandler, J. M., Jan, C. & Beard, B. H. *Syst. Bot.* **11**, 353–371 (1986).
7. Heiser, C. B. *Rhodora* **60**, 271–283 (1958).
8. Heiser, C. B., Smith, D. M., Clevenger, S. & Martin, W. C. *Mem. Torrey bot. Club* **22**, 1–218 (1969).
9. Rieseberg, L. H., Beckstrom, S., Liston, A. & Arias, D. *Syst. Bot.* **16**, 50–76 (1991).
10. Rieseberg, L. H. *Am. J. Bot.* **78**, 1218–1237 (1991).
11. Heiser, C. B. *Evolution* **1**, 249–262 (1947).
12. Nabhan, G. P. & Reichardt, K. L. *SW. Nat.* **28**, 231–235 (1983).
13. Crawford, D. J. *Plant Molecular Systematics* (Wiley, New York, 1990).
14. Williams, J. K. G., Kubelick, A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. *Nucleic Acids Res.* **18**, 6531–6535 (1990).
15. Rieseberg, L. H., Choi, H. C., Chan, R. & Spore, C. *Heredity* **70**, 285–293 (1993).
16. Lander, E. S. et al. *Genomics* **1**, 174–181 (1987).
17. Holm, D. G., Fitz-Earle, M. & Sharp, C. B. *Theor. appl. Genet.* **57**, 247–256 (1980).
18. Coyne, J. A. *Evolution* **28**, 505–506 (1974).
19. Rieseberg, L. H., Carter, R. & Zona, S. *Evolution* **44**, 1498–1511 (1990).
20. Goodfellow, P. N. *Curr. Biol.* **3**, 149–151 (1993).
21. Fritsch, P. & Rieseberg, L. H. *Nature* **359**, 633–636 (1992).

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Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene

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ERYTHROID Krüppel-like factor (EKLF) was originally isolated from erythroid cell RNA by differential screening and shown to be erythroid-specific, although a low level of EKLF was found in mast cell lines^{1,2}. EKLF contains three zinc-fingers homologous to those found in the Krüppel family of transcription factors. Because it binds the sequence CCACACCCT, EKLF may affect erythroid development as a result of its ability to bind to the CAC box in

the promoter of the β -globin gene^{1,2}. Mutation of this element leads to reduced β -globin expression^{3–5} and it appears to mediate the effect of the globin locus control region on the promoter⁶. Here we inactivate the EKLF gene through insertion of a *lacZ* reporter gene by homologous recombination in embryonic stem (ES) cells. Heterozygous EKLF^{+/-} mice show that the reporter gene is expressed in a developmentally specific manner in all types of erythroblasts in the fetal liver and adult bone marrow. Homozygous EKLF^{-/-} mice appear normal during the embryonic stage of haematopoiesis in the yolk sac, but develop a fatal anaemia during early fetal life when haematopoiesis has switched to the fetal liver. Enucleated erythrocytes are formed but these do not contain the proper amount of haemoglobin. We conclude that the transcription factor EKLF is essential for the final steps of definitive erythropoiesis in fetal liver.

Using the known EKLF sequence¹, we isolated a cosmid from a 129 mouse DNA library and inserted a *lacZ* reporter gene⁷ in frame and a marker gene to select for neomycin resistance⁸ at the ATG start codon (Fig. 1). Transfection of the linearized plasmid into E14 embryonic stem cells⁹ and double selection (*neo*⁺, tk⁻)¹⁰ resulted in 60% homologous recombinants, as determined by Southern blot analysis of individual clones (Fig. 1). Six of the ES clones with a 70–90% normal karyotype were injected into mouse blastocysts¹¹, and three (D1, D8 and D32) resulted in chimaeric mice that transmitted the EKLF⁻ allele through the germ line (Fig. 1). The three founder lines were all bred to different background mice (FVB, BL6 and CBA). All of these combinations resulted in the same phenotype and therefore the different mice will not be discussed separately.

Analysis of the different developmental stages of the mice showed that the *lacZ* coding unit driven by the EKLF gene was first expressed in all types of erythroblasts (not shown) in the fetal liver (Fig. 2a) through to adult bone marrow. The gene was not expressed in the embryonic yolk sac or blood (as shown by the lack of LacZ staining, which does not exclude a low level of EKLF expression; Fig. 2b) or in any other tissue during

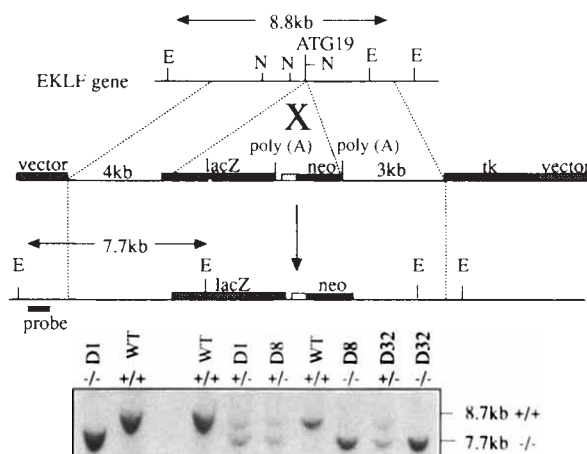
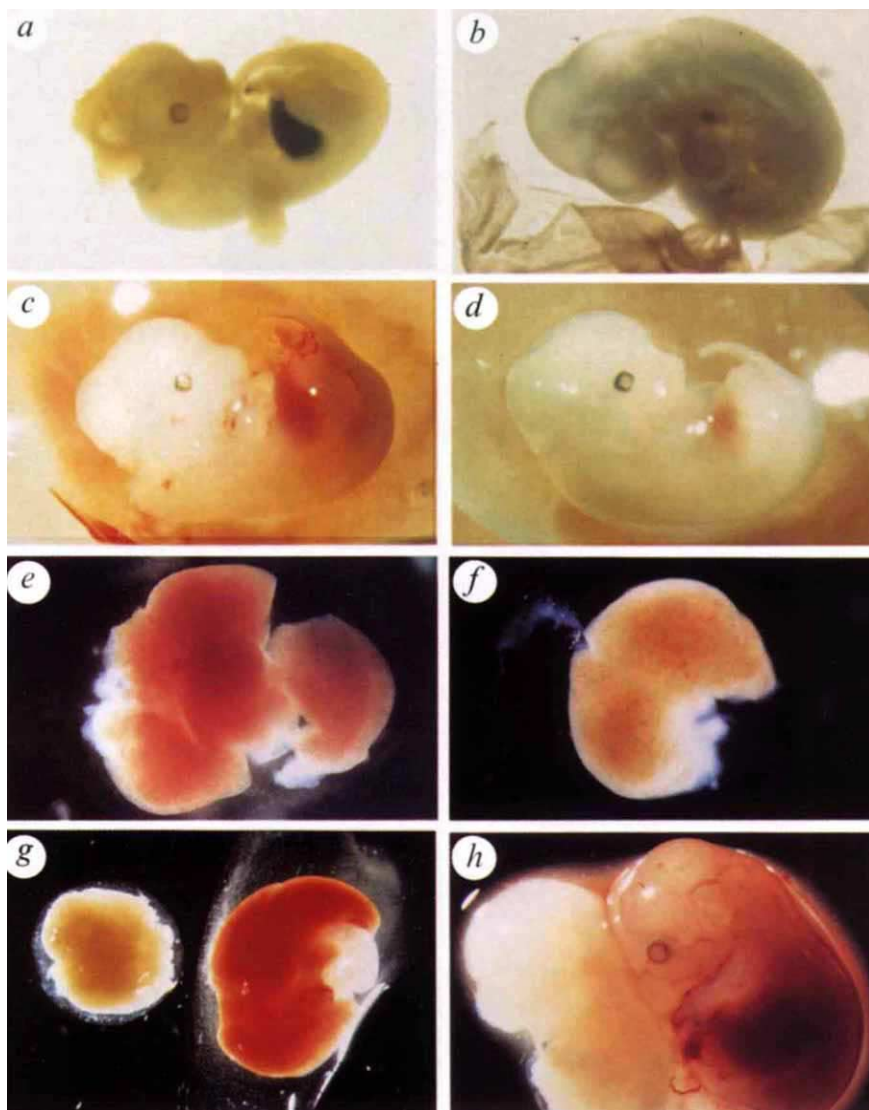


FIG. 1 Homologous recombination into the murine EKLF gene. The coding region of the bacterial *lacZ* gene⁷ and the *neo*-selectable marker¹⁰ were isolated as a 4.6-kb (*Nco*I compatible) *Bsp*HI fragment (D.M., unpublished work) and cloned into a partially digested 7-kb *Nco*I fragment at methionine 19 of the EKLF gene¹. The linearized EKLF/*lacZ*/*neo* plasmid, which already contained the herpes simplex virus thymidine kinase (tk) gene, was introduced into E14 ES cells⁹. Homologous recombinants were detected by hybridization with internal (*lacZ*) and external flanking probes. After creating chimaeric mice by ES cell injection into blastocysts¹¹, three separate founder lines were established. Bottom panel, Southern blot hybridization with a 5' flanking probe of the three founder lines. The wild-type (WT) EKLF allele is detected as an 8.7-kb fragment; the recombinant allele is detected as a 7.7-kb fragment, because of the introduction of a novel *Eco*RV site. E, *Eco*RV; N, *Nco*I.

FIG. 2 Analysis of EKLF/lacZ mice. *a*, LacZ staining²² of a heterozygous EKLF^{+/-} 12.5-day-old fetus. The fetal liver shows intense blue staining. Two blue spots below the back of the head are consistently observed in LacZ-expressing and non-expressing mice and are therefore not related to EKLF expression. *b*, LacZ staining of a homozygous EKLF^{-/-} 10.5-d embryo. The embryo shows no staining in the blood and is otherwise indistinguishable from +/- or +/+ control embryos (not shown). *c*, A 12.5-d EKLF^{+/-} fetus; note the normal colour of red cells containing haemoglobin. *d*, A 12.5-d EKLF^{-/-} fetus (littermate of fetus in *c*); note the pale liver. *e* and *f*, 13.5-d fetal liver from +/+ (red) and -/- (pale) littermates. *g*, 14.5-d EKLF^{+/-} and EKLF^{-/-} fetal livers from two littermates (*h*). Note the -/- liver has no haemoglobin and is much smaller than the +/- control. *h*, 14.5-d +/- and -/- littermates. The EKLF^{-/-} fetus (left) is severely anaemic compared to its +/- littermate.



development. The heterozygous mice appeared to be healthy, they were fertile and their haematocrit, mean corpuscular volume and absolute and differential blood cell counts were normal (not shown). Cell numbers in spleen and bone marrow were the same as in wild-type littermates.

Crossing of heterozygous EKLF^{+/-} animals did not result in live-born homozygous EKLF^{-/-} mice. At earlier stages, embryos at day 10.5 appeared normal (Fig. 2*b*), containing apparently normal nucleated embryonic (E10.5) erythrocytes in the yolk sac and blood (data not shown). But after switching of embryonic haematopoiesis to the fetal liver, the EKLF deletion gives rise to a severe phenotypic effect (Fig. 2*c*, *d*, *h*). The fetal liver contains normal numbers of the various erythroblasts, including enucleated cells that lack their full complement of haemoglobin, resulting in a rapid anaemia, erythroblastosis and growth retardation. The embryo dies around day 14, presumably because the remaining circulating blood cells can no longer sustain life (see Fig. 2*g*, *h* for example). Although the growth and development of the fetal liver is severely affected (Fig. 2*e-g*), a normal number of colony-forming cells were seen in the early fetal liver, indicating that the progenitor cell compartment¹² is not affected (about 3,000 CFU-C per fetal liver in EKLF^{-/-} and EKLF^{+/-} mice; data not shown). To confirm that the primitive (embryonic) blood cells, as opposed to the definitive (fetal liver) cells, are normal, we analysed the different β -globin-like genes using U6 or β -actin genes as a control. S1 nuclease protection

experiments (Fig. 3) show the embryonic $\epsilon\gamma$ and β -h1 RNAs to be present, with the expected persistence of the signals in the circulating embryonic blood in the fetus^{13,14}. However, only small amounts of mature β -maj RNA (<10-fold) can be detected in the fetal liver of EKLF^{-/-} mice, in agreement with the phenotype already described. The α -globin gene is clearly expressed in the embryonic yolk sac, fetal liver and blood of all mice. We have also probed for the expression of other erythroid genes (CA1 and PBGD) containing a CAC box with a single base EKLF consensus mismatch¹⁵⁻¹⁷, and both of these are expressed at high levels in the fetal liver, although the PBGD gene expression may be slightly reduced (data not shown).

We conclude that EKLF is essential for the last steps of differentiation of mature erythroid progenitors and that it is unlikely to be important in the early events that lead to red cell differentiation. It is not yet known which genes are directly regulated by EKLF, but its presence in definitive erythropoiesis (fetal liver and bone marrow) and absence (or low level¹⁸) in primitive erythropoiesis (embryonic yolk sac) and its expression pattern in the differentiation of red cells, all suggest that it targets the genes involved in haemoglobin production. Our results combined with those from transfection experiments^{1,2} indicate that EKLF could be involved in the expression of adult β -globin genes by specifically recognizing the CAC box in the fetal/adult stages. This CAC box is slightly different from those in the embryonic globin genes and from that in the α -globin genes.

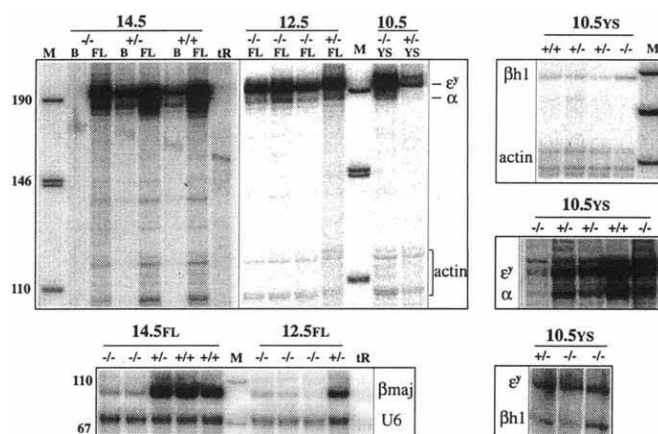


FIG. 3 Phosphorimage of S1 nuclease analysis²³ using probes for the mouse globin ϵ , β -h1, β -maj and α genes. Analysis of RNA samples from 10.5-d, 12.5-d and 14.5-d normal (+/+), EKLF^{+/-} or EKLF^{-/-} embryos and fetuses. B, blood; FL, fetal liver; YS, yolk sac + blood. The protected fragments ϵ (195 nt), β -h1 (180 nt), β -maj (100 nt), α (185 nt), U6 (75 nt) and β -actin (105 and 115 nt) are indicated. The numbers on the left indicate the size (in nucleotides) of the fragments in the marker lane (M). Top left, developmental time series of ϵ , α and actin RNA of EKLF +/+, +/- and -/- littermates. Each time point was a different experiment and the ratio of the specific activities of α and ϵ is different from that shown in the centre right panel (different litter). Blood samples contain very low levels of β -actin RNA; EKLF^{-/-} blood contains almost no RNA. tR indicates control lanes containing tRNA only. Bottom left, Fetal liver expression of β -maj and U6 RNA at 14.5 d and 12.5 d of development. Top right, β -h1 versus β -actin RNA. The level of β -h1 varies, as do all other embryonic signals owing to slight differences in the actual development time of each individual fetus²⁰. Centre right, ϵ versus α -globin RNA. The ratio of these varies to some extent, but EKLF^{-/-} embryos are not significantly different from littermates. Bottom right, ϵ versus β -h1 globin RNA.

The adult human β -globin gene (with the same CAC box as the mouse β -maj gene) can be expressed efficiently in the mouse embryonic yolk sac and blood (namely, in tissue lacking EKLF), both as a small LCR-containing construct¹⁹⁻²¹ and in the context of the complete human β -globin locus (N. Dillon and F.G., manuscript submitted). This suggests that EKLF is not required *per se* for β -globin gene expression and that a different factor may fulfil the role of EKLF in the embryonic yolk sac. After that stage, EKLF is probably essential, its absence resulting in a reduction of globin expression even more severe than that caused by β -globin CAC-box mutations^{3, 5}. □

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1. Miller, I. S. & Bieker, J. J. *Molec. cell. Biol.* **13**, 2776-2786 (1993).
2. Feng, W. C., Southwood, C. M. & Bieker, J. J. *J. biol. Chem.* **269**, 1492-1500 (1994).
3. Orkin, S. H. et al. *Nature* **296**, 627-631 (1982).
4. Orkin, S. H., Antonarakis, S. E. & Kazanian, H. H. *J. biol. Chem.* **259**, 8679-8681 (1984).
5. Kulozik, A. E., Bellan-Koch, A., Bail, S., Kohne, E. & Kleihauer, E. *Blood* **77**, 2054-2058 (1991).
6. Antoniou, M. & Grosfeld, F. *Genes Dev.* **4**, 1007-1013 (1990).

7. Kalderon, D., Roberts, B., Richardson, W. D. & Smith, A. E. *Cell* **39**, 499-509 (1984).
8. Thomas, K. R. & Capecchi, M. R. *Cell* **51**, 503-512 (1987).
9. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Moule, M. *Nature* **326**, 292-295 (1987).
10. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. *Nature* **336**, 348-352 (1988).
11. Robertson, E. J. *Teratocarcinomas and Embryonic Stem Cells* (IRL, Oxford and Washington DC, 1987).
12. Ploemacher, R. E., Van Soest, P. L., Boudewijn, A. & Neben, S. *Leukemia* **7**, 1374-1380 (1993).
13. Wong, P. M. et al. *Blood* **62**, 1280-1288 (1983).
14. Chada, K. et al. *Nature* **314**, 377-380 (1985).
15. Epperly, B. R., Berghem, N. C. H., Venta, P. J. & Tashian, R. E. *Gene* **131**, 249-253 (1994).
16. Beaumont, C., Porcher, C., Picat, C., Nordmann, Y. & Grandchamp, B. *J. biol. Chem.* **264**, 14829-14834 (1989).
17. Eleouet, J. F. & Romeo, P. H. *Eur. J. Biochem.* **272**, 763-770 (1993).
18. Donze, D., Townes, T. & Bieker, J. J. *J. biol. Chem.* **270**, 1955-1959 (1995).
19. Behringer, R. R., Ryan, T. M. & Palmiter, R. D. *Genes Dev.* **4**, 380-389 (1990).
20. Enver, T., Raich, N. & Ebens, A. S. *Nature* **344**, 309-313 (1990).
21. Hanscombe, O. et al. *Genes Dev.* **5**, 1387-1394 (1991).
22. Bonnerot, C. & Nicholas, J. F. *Meth. Enzym.* **225**, 451-469 (1993).
23. Strouboulis, J., Dillon, N. & Grosfeld, F. *Genes Dev.* **6**, 1857-1864 (1992).

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Lethal β -thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF

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GLOBIN genes are regulated in a tissue-specific and developmental stage-specific manner, with the β -globin gene being the last to be activated in the β -gene cluster¹. CACCC-nucleotide sequences, which bind multiple nuclear proteins, including ubiquitously expressed Sp1 and erythroid Krüppel-like factor (EKLF), are among the *cis*-regulatory sequences critical for transcription of globin and non-globin erythroid-expressed genes²⁻⁵. To determine the function of EKLF *in vivo*, we created mice deficient in EKLF by gene targeting⁶. These embryos die of anaemia during fetal liver erythropoiesis and show the molecular and haematological

features of β -globin deficiency, found in β -thalassaemia. Although it is expressed at all stages, EKLF is not required for yolk sac erythropoiesis, erythroid commitment or expression of other potential target genes. Its stage-specific and β -globin-gene-specific requirement suggests that EKLF may facilitate completion of the fetal-to-adult (haemoglobin γ to β) switch in humans.

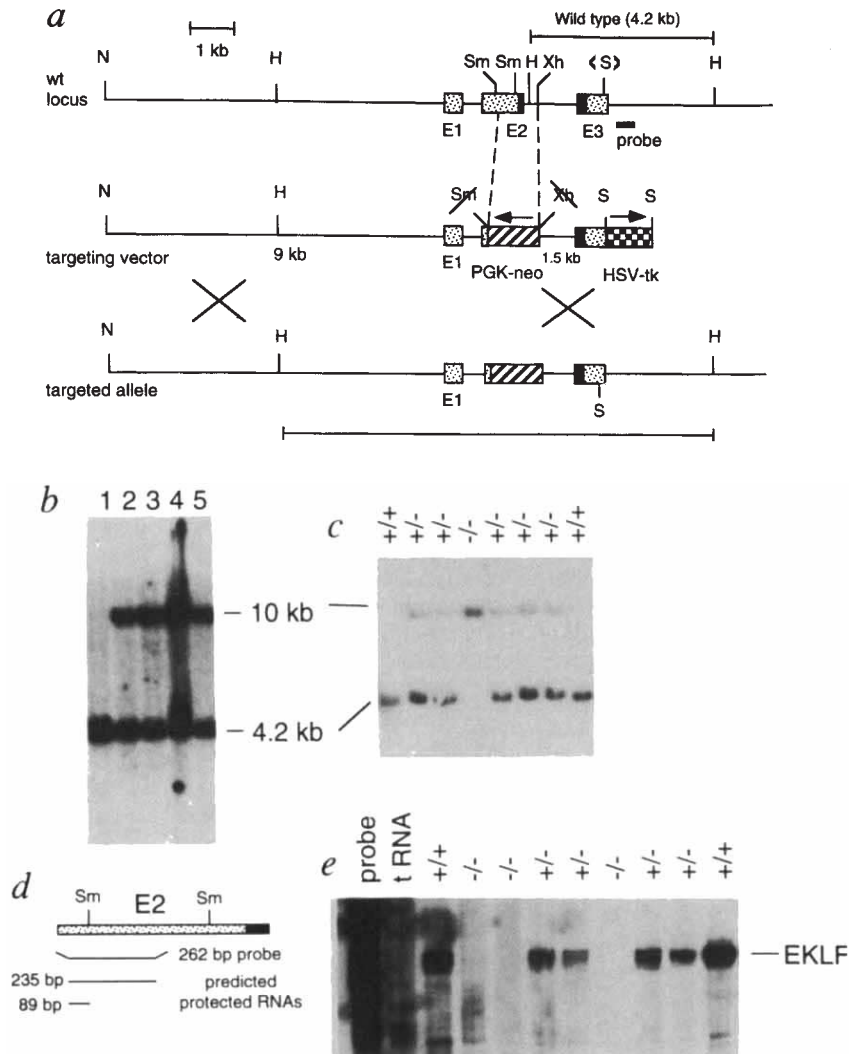
Using homologous recombination in embryonic stem (ES) cells, we inactivated the mouse EKLF gene by replacing a portion of the zinc-finger DNA-binding domain with a neomycin-resistance *neo*^R cassette (Fig. 1a). ES cells with a targeted allele (Fig. 1b) were used to generate heterozygous (EKLF^{+/-}) mice which were bred together to produce homozygous (EKLF^{-/-}) mice (Fig. 1c). Stable EKLF transcripts were not detected in EKLF^{-/-} embryos (Fig. 1d, e).

EKLF^{-/-} embryos appeared to be normal during the yolk sac stage, but became progressively more anaemic from embryonic-day 11 (E11) to E15, paralleling the dependence on the switch from embryonic to fetal-liver-derived erythropoiesis (Fig. 2a-e; Table 1). Heterozygous mice were unaffected. EKLF^{-/-} embryos died by E16. Peripheral blood of E15 EKLF^{-/-} embryos revealed residual embryonic red cells of normal appearance, but abnormally small and irregularly shaped red cells of fetal liver origin (Fig. 2d-e). Increased numbers of nucleated fetal liver erythroblasts were also present in the circulation. Fetal livers of EKLF^{-/-} embryos were of normal size and replete with erythroid precursors (see below and Table 1), but erythroid maturation, notably haemoglobin accumulation, was defective

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FIG. 1 Generation of mice with a targeted mutation of the EKLF gene. **a**, Schematic representations of the wild-type EKLF locus, the targeting vector, and the predicted targeted allele are shown. Stippled boxes represent exons. Black boxes represent regions encoding the zinc-finger domains. Hatched and checked boxes depict phosphoglycerate kinase (PGK)-neo and herpes simplex virus thymidine kinase (HSV-tk) cassettes, respectively. Arrows indicate transcriptional orientation. A 3'-flanking probe was used to screen for homologous recombination events (**b** and **c**). Abbreviations: N, NotI; H, HindIII; Sm, SmaI; S, SalI; Xh, XhoI. The restriction map is complete only for HindIII sites. Crossed letters represent restriction sites destroyed in cloning. The bracketed Sal site in the wild-type locus, which was used in the plasmid construction, was artificial and present in the linker of an EKLF bacteriophage clone. **b**, Southern analysis of genomic DNA from five selected ES cell clones. Hybridization of the probe to 4.2-kb (wild type) and 10-kb (homologous recombinant) genomic HindIII fragments is indicated. **c**, Southern analysis of a litter (killed at E15) generated from an EKLF^{+/-} cross. Wild type (+/+), heterozygous (+/-) and homozygous (-/-) embryos were present in the litter. **d**, Diagram of the EKLF riboprobe. Exon2 (E2) is represented. The 262-bp riboprobe (including plasmid sequences) was predicted to protect an intact EKLF RNA species of 235 bp. If stable RNA were present that had arisen from the targeted allele, an 89-bp fragment would be expected. **e**, Absence of EKLF transcripts in EKLF^{-/-} embryos (killed at E15). RNA loadings were equivalent, as verified by hybridization with a GATA-1 riboprobe (not shown). No 89-bp protected RNA was seen in either EKLF^{+/-} or EKLF^{-/-} samples.

METHODS. EKLF genomic clones were isolated by standard methods from a λ FixII mouse strain-129 library (Stratagene)²². Partial restriction mapping, sequencing and using analysis polymerase chain reaction (PCR) were performed to define exon/intron boundaries. A portion of exon 2 and intron 2 lying between SmaI and XhoI sites was replaced by a PGK-promoter neo^r cassette. An HSV-thymidine kinase cassette was included at the boundary of the targeting construct to provide for negative selection²³. A 3'-flanking probe was generated by PCR labelling with the primers CTGTGCCATCCGCTAGGATTG and GTTCAGGCCAAC-CAGGGCTAC. J1 EL cells²⁴ were electroporated with NotI-linearized targeting construct, selected in G418 (250 μ g ml⁻¹) and FIAU (2 μ M) (Lilly), and expanded for Southern analysis. Targeted clones were injected into C57BL/6 blastocysts, which were transferred to the uterus in pseudo-pregnant BALB/c Swiss mice. Male chimaeras were bred against the



background of the host blastocyst. The genotypes of live-born animals and of the litters killed *in utero* were determined by Southern blot analysis of genomic DNA from tails and carcasses, respectively. To construct an EKLF riboprobe, nucleotides 481–716 of EKLF cDNA were amplified using two primers (AP1, ggattccgctccgaggagcacaca and AP3, gctctagagctgtgtggcgggggtaca)⁴. The 235-bp product, which spans the SmaI site in exon 2, was cloned into the SrfI site of pCR-Scribe (Stratagene) and sequenced to determine orientation. RNase protection of EKLF transcripts was assayed as described²⁵.

(Fig. 2d, e). Electron microscopy of EKLF^{-/-} fetal liver erythroid cells revealed inclusions typical of precipitated globins, as well as erythrophagocytosis by Kupffer cells (not shown). Stainable iron in Kupffer cells and erythroblasts (siderocytes) was appreciably increased (Fig. 2f, g), consistent with ineffective erythropoiesis and a feature of the thalassaemia syndromes⁷.

To delineate the pattern of globin gene expression in the absence of EKLF, we analysed fetal and yolk sac RNA samples for embryonic and adult globin transcripts. As shown in Fig. 3a, adult β -globin RNA was markedly decreased (~10-fold) relative to α -globin or GATA-1 (ref. 8) transcripts in E15 EKLF^{-/-} fetal liver. Accordingly, β -globin protein was dramatically reduced relative to α -globin (~20-fold) in blood samples (Fig. 3b). Thus, mutant mice shared an imbalance of their globin chains typical of thalassaemia⁷. Haematopoietic colony assays of fetal liver progenitors revealed normal numbers of granulocyte/macrophage and mast cell colony-forming units (CFU) (Table 1). Moreover, although the numbers of erythroid precursors (BFU-e and CFU-e) were normal, they failed to mature properly

and were markedly deficient in β -globin RNA (not shown), mirroring the deficit observed in intact embryos. Based on the crystal structure of a related zinc-finger protein, EKLF was predicted to bind to an extended CACCC-box consensus (CCNCNCCCN, where N is any nucleotide⁴) which is found in the promoters of several erythroid-expressed genes. Despite the presence of a consensus EKLF-type CACCC motif in the embryonic globin β h1 promoter, its expression, and that of other embryonic globins (ϵ and ζ), was normal (Fig. 3c). Of other known erythroid genes with consensus EKLF-type CACCC sites in their promoters, GATA-1 (ref. 9) (Fig. 3a), erythropoietin receptor¹⁰ (EpoR; not shown), and porphobilinogen deaminase (PBGD)¹¹ (Fig. 3d) were also normally expressed. Thus, despite the presence of putative consensus sites in other erythroid genes, EKLF loss leads to a highly selective deficiency of β -globin expression.

The dependence of β -globin expression on EKLF is consistent with prior independent data showing that EKLF transactivates β -globin promoter constructs and fails to bind or activate pro-

motors containing β -thalassaemia mutations^{4,12}. Although factors present in non-erythroid cells, such as the ubiquitous factor Sp1, are able to function at the β -globin promoter CACCC-site², our data reveal an unexpected degree of *in vivo* specificity for EKLF action. Both the binding affinity of EKLF to particular CACCC sequences and promoter context are likely to contribute to this selectivity. For example, we observe differing affinities of binding of particular consensus sequences to recombinant EKLF *in vitro*, and yet the CACCC motifs of the β -globin and PBGD promoters are bound with comparably high affinity (data not shown).

The stage-specific requirement raised the possibility that EKLF is either not expressed or is inactive during yolk sac erythropoiesis. By RNase protection assay, however, EKLF transcripts were as abundant in purified yolk sac erythroid cells as in fetal liver erythroid cells (not shown). The CACCC-binding activity, corresponding to CAD-D of ref. 5, was similar in embryonic and definitive erythroid cells (relative to Sp1-binding activity), but selectively missing in cells of EKLF^{-/-} mice (Fig. 3e). To assess EKLF activity in embryonic cells, we assayed purified E11 yolk sac erythroid cells of EKLF^{-/-}, EKLF^{+/-} and wild-type origins for β -globin transcripts. Critical to our analysis is the recognition that yolk-sac-derived erythroid cells switch on expression of small amounts of β -globin by this time¹³. As shown in Fig. 3f, E11 EKLF^{-/-} embryonic red cells, although phenotypically normal, contained far fewer β -globin transcripts than

TABLE 1 Haematology and fetal liver progenitor cell analysis at E15

Genotype	HCT (%)	Hb (g dl ⁻¹)	Progenitor cell numbers $\times 10^{-3}$ per fetal liver*			
			BFU-e	CFU-e	CFU-mast	CFU-GM
+/(n=5)	45 $\pm$ 8	8 $\pm$ 1.1	10.1 $\pm$ 2.5	191 $\pm$ 45	10.3 $\pm$ 4.5	3.3 $\pm$ 0.7
+/(n=18)	40 $\pm$ 7.3	7 $\pm$ 2.3				
-/(n=5)	8 $\pm$ 1.8	0.4 $\pm$ 0.3	9.5 $\pm$ 3.4	207 $\pm$ 23	7.9 $\pm$ 3.7	3.0 $\pm$ 0.3

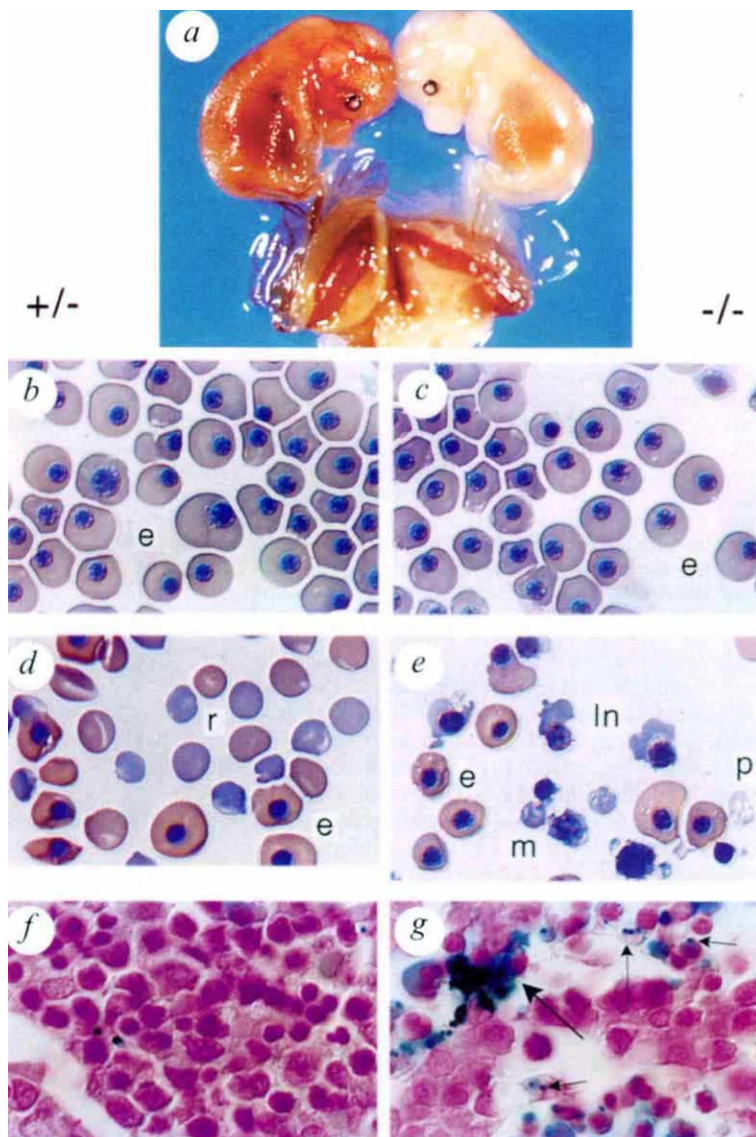
Haematocrits were determined on 2 μ l of blood using microhaematocrit tubes (Microcaps, Drummond Scientific). Haemoglobin concentration was determined by absorbance at 570 nm in a spectrophotometer after its conversion to cyanmethaemoglobin in Drabkin's solution. The numbers of haematopoietic progenitors were determined by standard methylcellulose assay.

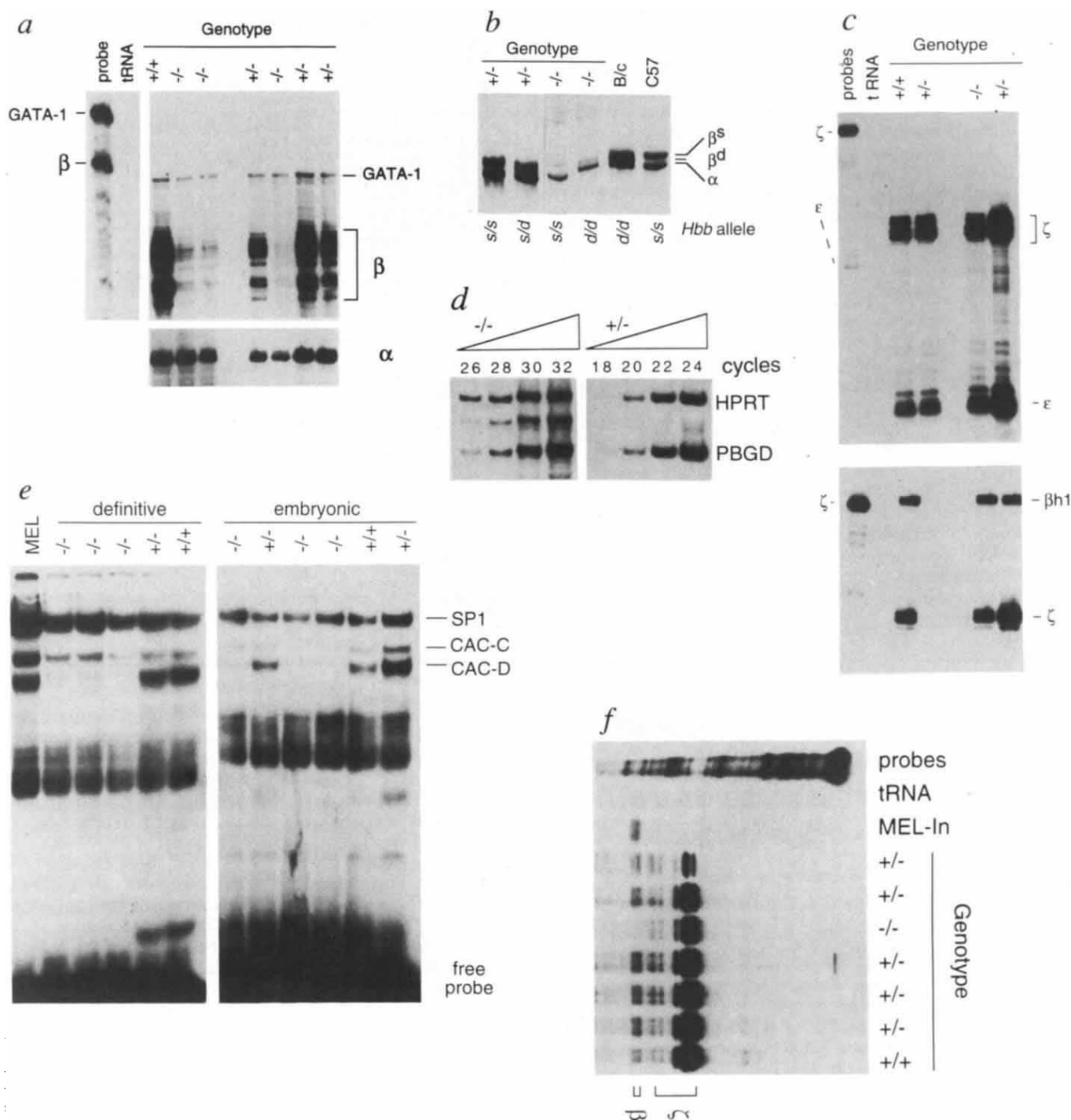
*Results derived from one wild-type and three EKLF^{+/-} embryos have been combined and compared with those from four EKLF^{-/-} embryos.

EKLF^{+/-} or wild-type cells. From this we conclude that EKLF is indeed functional in embryonic cells, and that the β -globin gene is likely to be a direct target for EKLF. Correlation of the *in vitro* DNA-binding specificity of EKLF with its ability to transactivate a β -globin reporter gene containing CACCC-box mutations in patients with thalassaemia is consistent with our latter inference¹². The stage-specific lethality in EKLF^{-/-} mice reflects the requirement for β -globin chain production to balance α -globin chain production in fetal liver-derived erythrocytes.

FIG. 2 Phenotype of EKLF^{-/-} embryos, erythrocytes and fetal liver. **a**, Photograph of EKLF^{-/-} and EKLF^{+/-} embryos at E15. The EKLF^{-/-} embryo is pale but otherwise developmentally normal. A marked deficit of red cells in the vitelline and umbilical circulations is evident. **b–e**, May–Grunwald–Geimsa-stained blood smears from EKLF^{+/-} (**b** and **d**) and EKLF^{-/-} (**c** and **e**) littermates killed at E11 (**b**, **c**) and E15 (**d**, **e**). Embryonic cells (**e**) are normal in the EKLF^{-/-} embryos (compare **c** with **b**), but definitive cells (**r**) are markedly abnormal (compare **e** with **d**). Microcytes (**m**), poikilocytes (**p**) and increased numbers of fetal liver-derived late normoblasts (**ln**) with irregular, poorly haemoglobinized cytoplasm are evident in **e**. Original magnification, $\times 400$. **f**, **g**, Prussian-blue-stained fetal liver sections. Haemosiderin within Kupffer cells (large arrow) and siderotic granules within developing normoblasts (small arrows) are evident in the EKLF^{-/-} (**g**) but not EKLF^{+/-} (**f**) fetal liver. Original magnification, $\times 400$.

METHODS. Embryos were removed on days E11–E15 (the morning of vaginal plug discovery was taken as E0) and dissected free of maternal tissue without disrupting the placenta or visceral yolk sac. For histological analysis, embryos were fixed in 4% buffered formalin, processed and embedded in paraffin. 5- μ m-thick sections were stained in haematoxylin and eosin or in Prussian blue, followed by safronin counter-staining. Blood smears and cytocentrifuge specimens were fixed in methanol and stained in May–Grunwald–Geimsa. Histological sections and blood films were photographed under Nomarski optics.





blood of embryos (killed at E15) generated by breeding of EKLF heterozygotes. Haemolysates from adult BALB/c (B/c) and C57BL/6 (C57) mice are included to serve as controls for the migration of the two different alleles (*Hbb^d* and *Hbb^s*) represented within the progeny of this cross. Marked chain imbalance due to β -globin deficiency is evident in the EKLF^{-/-} embryos, irrespective of which *Hbb* allele was inherited. c, RNase protection analysis of embryonic ζ , ϵ , β h1 globin RNAs in E11.5 yolk sac erythroid cells. d, Semi-quantitative reverse transcription (RT)-PCR analysis of porphobilinogen deaminase (PBGD) expression in EKLF^{-/-} fetal liver erythroid cells. Inclusion of primers specific for hypoxanthine phosphoribosyl transferase (HPRT) transcripts served as an internal control for quantification. The ratio of HPRT to PBGD is similar in EKLF^{-/-} and EKLF^{+/+} erythroid cells. The number of amplification cycles is shown. e, Gel-shift CACCC-binding activity in nuclear extracts of definitive (fetal liver-derived) and embryonic erythroid cells. The genotypes of the dead embryos are indicated. Sp1 was assigned based on supershift with specific antiserum (not shown). CAC-C and CAC-D were assigned according to ref. 5. Absence of the complex previously designated CAD-D⁵ correlates with the EKLF^{-/-} genotype. Mouse erythroleukaemic (MEL) cell nuclear extract is provided as a positive control. f, RNase protection analysis of β - and ζ -globin transcripts in E11.5 yolk sac erythroid cell RNA. MEL-In, MEL cells induced to differentiate for 48 h in dimethylsulphoxide. Note the marked reduction in β -globin transcripts in the EKLF^{-/-} embryo.

METHODS. Total RNA was prepared from cell suspensions and fetal livers²⁶. Murine β -globin, α -globin, ζ -globin, ϵ -globin, GATA-1 and γ -actin transcripts were measured by RNase protection²⁵ or by RT-PCR²⁷. To generate a riboprobe for β h1 globin, cDNA was amplified by RT-PCR with specific primers: AP17, ACCTCACAC-CATGGTTCACTTCAC and AP18, AGTTTAATGGTACTTGTGGGAAGA. The PCR product was cloned into the SrfI site of pCR-Scribe (Stratagene) and sequenced to determine orientation. For globin polypeptide analysis, whole blood (1 μ l) was lysed in 5 $\times$ volume of 66 mM cystamine, and globin chains were separated on a 13% polyacrylamide gel containing 0.5% Triton X-100, 8M urea and buffered in 5% acetic acid²⁸. Gels were stained in Coomassie blue and the protein in individual bands quantified as described²⁸. Murine PBGD transcripts were estimated by semiquantitative RT-PCR²⁷ using a primer pair corresponding to sequences in the erythroid-specific exon 2 (AP45, AACCTCCTTGGCCAGGCTGGGCTT) and exon 3 (AP46, TCTTACGGGT-ACCCTCTCGAATCA)¹¹. Primer pairs used to amplify HPRT and EpoR have been described²⁷. Nuclear extracts were prepared from yolk-sac-derived embryonic or fetal liver-derived definitive erythroid cells²⁹. The gel-shift probe was a double-stranded oligonucleotide corresponding to the β -globin CACCC site (tagagCCACACCTTggaag). Binding was determined in the presence of 1 μ g of nonspecific competitor DNA (poly dI.dC) and 0.2 ng of ³²P end-labelled probe⁸.

Our findings have important implications for globin gene regulation. First, they establish the β -globin gene as a selective and probably direct *in vivo* target gene for EKLF. Normal expression of other potential targets, such as the β h1-globin gene, argues that EKLF interacts specifically with components of the β -gene promoter and/or the globin-locus control region. Second, the surprisingly selective effect of EKLF loss on β -globin transcription raises the possibility that mutations in the EKLF gene might account for rare cases of thalassaemia genetically unlinked to the β -gene cluster¹⁴, particularly as substitutions in EKLF-binding sites in the β -globin promoter cause β -thalassaemia¹⁵. Third, in contrast to the erythroid transcription factors GATA-1 and tal-1/SCL^{16,17}, EKLF is dispensable for erythroid commitment or the proliferation of erythroid precursors. Finally, as a factor dedicated to β -globin gene expression, EKLF may participate in globin gene switching. Although expression of human fetal γ -genes is substantially shut down during development of transgenic mice in the absence of a linked β -globin gene¹⁸⁻²⁰, EKLF may facilitate completion of the fetal-to-adult switch. Accordingly, after the initial silencing of γ -globin gene expression, interactions of the β -globin promoter with EKLF may help to reduce γ -globin transcription further, perhaps by competition for the globin locus control region. This view is compatible with the consistent increase in γ -globin expression that accompanies deletions of the β -gene promoter and point mutations in the β -globin CACCC site²¹. We therefore suggest that selective inhibition of EKLF might assist therapeutic strategies designed to maximize γ -globin expression in adults with thalassaemia or other haemoglobinopathies. □

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1. Crossley, M. & Orkin, S. H. *Curr. Opin. Genet. Dev.* **3**, 232-237 (1993).
2. Treisman, R., Orkin, S. H. & Maniatis, T. *Nature* **302**, 591-596 (1983).
3. Myers, R. M., Tilly, K. & Maniatis, T. *Science* **232**, 613-618 (1986).
4. Miller, I. J. & Bieker, J. J. *Molec. cell. Biol.* **13**, 2776-2786 (1993).
5. Hartzog, G. A. & Myers, R. M. *Molec. cell. Biol.* **13**, 44-56 (1993).
6. Capecchi, M. R. *Science* **244**, 1288-1292 (1989).
7. Nathan, D. G. & Gunn, R. B. *Am. J. Med.* **41**, 815-830 (1966).
8. Tsai, S.-F. *et al. Nature* **339**, 446-451 (1989).
9. Tsai, S.-F., Strauss, E. & Orkin, S. H. *Genes Dev.* **5**, 919-931 (1991).
10. Youssoufian, H., Zon, L., Orkin, S. H., D'Andrea, A. D. & Lodish, H. F. *Molec. cell. Biol.* **10**, 3675-3682 (1990).
11. Porcher, C. *et al. J. biol. Chem.* **266**, 10562-10569 (1991).
12. Feng, W. C., Southwood, C. M. & Bieker, J. J. *J. biol. Chem.* **269**, 1493-1500 (1994).
13. Brotherton, T. W., Chui, D. H. K., Gauldie, J. & Patterson, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2853-2857 (1979).
14. Thien, S. L., Wood, W. G., Wickramasinghe, S. N. & Galvin, M. C. *Blood* **82**, 961-967 (1993).
15. Orkin, S. H. *et al. Nature* **296**, 627-631 (1982).
16. Pevny, L. *et al. Nature* **349**, 257-260 (1991).
17. Shivdasani, R., Mayer, E. & Orkin, S. H. *Nature* **373**, 432-434 (1995).
18. Dillon, N. & Grosfeld, F. *Nature* **350**, 252-254 (1991).
19. Lloyd, J. A., Krakowsky, J. M., Crable, S. C. & Lingrel, J. B. *Molec. cell. Biol.* **12**, 1561-1567 (1992).
20. Stamatoyannopoulos, G., Josephson, B., Zhang, J.-W. & Li, Q. *Molec. cell. Biol.* **13**, 7636-7644 (1993).
21. McDonagh, K. T. & Nienhuis, A. W. in *Hematology of Infancy and Childhood* 4th Edn (eds Nathan, D. G. & Oski, F. A.) 783-789 (Saunders, Philadelphia, 1992).
22. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
23. Mansour, S., Thomas, K. & Capecchi, M. *Nature* **336**, 348-352 (1988).
24. Li, E., Bestor, T. H. & Jaenisch, R. *Cell* **69**, 915-926 (1992).
25. Baron, M. H. & Maniatis, T. *Cell* **46**, 591-602 (1986).
26. Chomczynski, P. & Sacchi, N. *Analyst. Biochem.* **162**, 156-159 (1987).
27. Weiss, M. J., Keller, G. & Orkin, S. H. *Genes Dev.* **8**, 1184-1197 (1994).
28. Alter, B. P. & Campbell, A. S. *Hemoglobin* **6**, 517-522 (1982).
29. Andrews, N. C. & Faller, D. V. *Nucleic Acids Res.* **19**, 2499 (1991).

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Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants

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THE identity and patterning of ventral cell types in the vertebrate central nervous system depends on cell interactions¹. For example, induction of a specialized population of ventral midline cells, the floor plate, appears to require contact-mediated signalling by the underlying notochord, whereas diffusible signals from the notochord and floor plate can induce ventrolaterally positioned motor neurons. Sonic hedgehog (Shh), a vertebrate hedgehog-family member, is processed to generate two peptides (*M*, 19K and 26/27K) which are secreted by both of these organizing centres^{2,30}. Moreover, experiments in a variety of vertebrate embryos³⁻⁵, and in neural explants *in vitro*⁵, indicate that Shh can mediate floor-plate induction. Here we have applied recombinant Shh peptides to neural explants in serum-free conditions. High concentrations of Shh bound to a matrix induce floor plate and motor neurons, and addition of Shh to the medium leads to dose-dependent induction of motor neurons. All inducing activity resides in a highly conserved amino-terminal peptide (*M*, 19K). Moreover, antibodies that specifically recognize this peptide block induction of motor neurons by the notochord. We propose that Shh acts as a morphogen to induce distinct ventral cell types in the vertebrate central nervous system.

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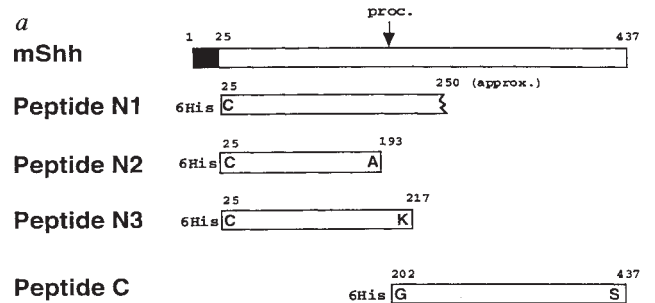
Considerable evidence indicates that conserved families of signalling molecules regulate pattern in both *Drosophila* and vertebrate development. One such family consists of members related to the segment polarity gene *hedgehog* (*hh*)⁶, which is important in embryonic and imaginal disc patterning⁷⁻¹⁷; *hh* encodes a precursor protein which undergoes autocleavage to generate amino and carboxyl peptides of relative molecular mass 19,000 and 25,000 (*M*, 19K and 25K), respectively¹⁶. Both peptides are secreted, although the 25K peptide appears to be more diffusible¹⁶. On the basis of this observation, and the analysis of phenotypes generated following heat-shock induction of wild-type and cleavage-deficient *Hh*, it has been proposed¹⁶ that each peptide may mediate distinct signalling functions, the 19K form short range, and the 25K form distant.

Several vertebrate *hh* homologues have been identified^{3-5,18,19}. One of these, *Sonic hedgehog* (*Shh*), has attracted considerable interest as it is expressed in four key embryonic signalling centres: the node, notochord, floor plate and zone of polarizing activity (ZPA) in the limb^{3-5,18,19}. Like *Drosophila* *Hh*, chick and mouse *Shh* are processed to generate distinct secreted forms, a 19K amino peptide which is retained at or near the cell surface, and a carboxyl 26/27K (chick/mouse) glycosylated peptide which is more diffusible². Sequence comparison among all the different hedgehog family members indicates that the 19K peptide corresponds to the most highly conserved region³, whereas the carboxyl peptide contains a putative catalytic site which is required for autocleavage¹⁶.

To investigate the possible ventralizing activity of *Shh* in the vertebrate central nervous system (CNS), we purified bacterially expressed peptides encompassing the mature forms of mouse *Shh* (Fig. 1a). The cleavage that generates the mature amino and carboxyl peptides *in vivo* occurs between amino acids 194 and 203 of mouse *Shh* (ref. 2), probably just before cysteine 199 (ref. 20). Peptide N1 includes the entire amino peptide and approximately 9K from the N-terminal end of the carboxyl peptide. Peptide N2 extends to amino acid 193. This form migrates in a similar position to the normal 19K cleavage product, when

FIG. 1 a, Bacterially expressed Sonic hedgehog peptides used in this study. A diagram of mouse Sonic hedgehog (mShh) indicating the signal peptide (filled box) and putative proteolytic processing site (proc.). The positions of the histidine tag (6His) in these peptides are indicated numerically with reference to the numbering scheme of Echelard *et al.*³. The precise C terminus of N1 has not been determined. b, Summary of floor-plate and motor-neuron induction by Shh peptides. Nickel-agarose beads saturated with Shh peptides were placed in contact with intermediate neural explants in culture, then scored for induction of ventral neural markers. Numbers represent number of explants that showed induction of each marker. Explants were scored as positive for Islet-1 induction when the number of induced cells was at least 10-fold higher than background levels. Of 19 control explants, only 5 showed any Islet-1 expression, and the maximum number of expressing cells observed was 24. In contrast, the minimum number of cells expressing Islet-1 observed in an Shh-induced explant was 230.

METHODS. DNA fragments containing mouse *Shh* coding sequences starting at amino acid 25 and extending to amino acid 437, 217 or 193 (ref. 3) were cloned between the *NdeI* and *XhoI* sites of pET102⁺ so that the *Shh* coding sequences were downstream of sequences encoding 6 consecutive histidine residues and a recognition sequence for the restriction protease thrombin. Transformed bacteria (BL21[DE3]plysS) were grown to A₅₅₀ of 0.6 and induced for 3 h with 0.5 mM IPTG. Bacterial pellets were lysed by sonication in lysis buffer (20 mM Tris-HCl (pH 7.9); 0.5 M KCl; 10% glycerol; 50 mM 2-mercaptoethanol) containing 0.1% NP-40, and the lysates were cleared by centrifugation at 15,000g for 20 min. The cleared lysates were mixed for 2 h at 4 °C with 0.1 vol of a 50% (by volume) slurry of nickel-agarose (Qiagen, Inc.) which had been equilibrated in lysis buffer. After washing the beads several times with lysis buffer containing 20 mM Imidazole, purified proteins were eluted with 250 mM Imidazole. Fractions containing large amounts of purified proteins (as judged by Coomassie blue staining of SDS-PAGE gels) were combined. Protein concentrations were determined by Bradford assays. After exhaustive dialysis against lysis buffer, purified peptides were mixed with small amounts of nickel-agarose for several hours. The beads were washed several times and samples were subjected to SDS-PAGE. The extent of protein coupling was assessed by Coomassie staining. This figure summarizes data obtained with peptide-saturated beads. Stage-10 chick embryos²¹ (8–10 somites) were incubated in 0.5 mg ml⁻¹ of dispase (Boehringer



b

	Beads					
	Notochord	Buffer	N1	N2	N3	C
<i>Shh</i> mRNA	21/25 (84%)	0/15	9/21 (43%)	nd	14/25 (56%)	0/9
SC-1/BEN	7/8 (88%)	0/6	12/12 (100%)	nd	nd	nd
Islet-1	18/19 (95%)	0/35	35/37 (95%)	0/16	12/15 (80%)	0/14

Mannheim) in DMEM (Sigma) for 2–5 min and the intermediate neural plate adjacent to the unsegmented mesoderm was isolated. Careful dissection avoided any floor plate or ventral cells, as well as any roof plate or dorsal cells. The dissected neural tissues were allowed to recover in DMEM containing 1% heat-inactivated horse serum for 5 min. Intermediate neural explants measured 300–400 µm along the rostro-caudal axis and 150–200 µm along the dorso-ventral axis. Notochords from stage 6/7 chick embryos were dissected using the same procedure. Tissue recombinants were embedded within three-dimensional collagen gels (Collaborative Biomedical Research) and cultured in 500 µl serum-free F12 medium (Gibco BRL) supplemented with N2 (Gibco BRL) containing antibiotics, at 37 °C in a 5% CO₂ environment for 48 h. Immunostaining and whole-mount *in situ* hybridization were performed using standard procedures.

expressed in tissue culture cells, but is missing a few amino acids at the C terminus which function to restrict diffusion from expressing cells². Peptide N3 extends to amino acid 217, and therefore encompasses the complete amino peptide, whereas peptide C initiates at amino acid 202 and therefore encompasses most of the mature carboxyl peptide.

Peptides were coupled to nickel-agarose beads, by an N-terminal hexahistidine tag, until the beads were saturated with protein. Several beads were then placed in contact with intermediate neural-plate explants, which had been isolated from stage-10 chick embryos²¹ and embedded in a collagen matrix. Explants were cultured for 48 hours in serum-free medium, then examined for the expression of ventral markers by whole-mount *in situ* hybridization (*Shh* RNA, floor plate marker¹⁸; *Islet-2* RNA, motor neuron marker²²) or immunostaining (SC-1/BEN, floor plate and motor neuron marker^{23,24}; *Islet-1*, motor neuron marker^{22,25}).

Ventralizing activity was restricted to peptides overlapping the mature 19K form of Shh. No induction was observed in response to peptides N2 or C, or to buffer-soaked beads (Figs 1b and 2b, g). In contrast, peptides N1 and N3, which encompass the complete amino peptide, induced *Shh*, SC-1/BEN, *Islet-1* and *Islet-2* expression in explants (Figs 1b and 2c–f, h–j; data not shown) as expected for induction of floor plate and motor neurons. Coexpression of *Islet-1* and SC-1/BEN (Fig. 2f), as well as expression of *Islet-2* (data not shown), provided unambiguous evidence of early motor-neuron development. Moreover, as SC-1/BEN immunostaining was clearly visible in extending axonal processes (Fig. 2e), at least some of these cells formed differentiated neurons. Expression of ventral markers was at least as strong as control induction using notochord isolated from stage

6/7 chick embryos²¹ (Fig. 2a; and data not shown). Whereas induction of *Shh* expression was generally restricted to the region of the explant in contact with the beads (Fig. 2h), *Islet-1* and SC-1/BEN expression was widely dispersed in the explants (Fig. 2c, d), and induction did not require contact, resembling results previously obtained with notochord grafts²⁶. Thus it is likely that either a high concentration of Shh peptide or a matrix-bound form is necessary to elicit induction of floor plate. The position and extent of motor-neuron induction could be modified by varying the amount of peptide coupled to the beads, although the results cannot be strictly quantified. Subsaturating levels of Shh peptide (N1 or N3) led to induction of fewer cells expressing both SC-1/BEN and *Islet-1*, and induced cells were found immediately adjacent to the bead (Fig. 2i, j).

Although 80% (N3) to 95% (N1) of explants showed strong induction of *Islet-1* positive motor neurons, only 43% (N1) to 56% (N3) showed *Shh* expression (Fig. 1b), suggesting that in some explants induction of motor neurons occurs in the absence of an induced floor plate. Thus, in these examples, induction of motor neurons does not appear to be a secondary consequence of signals derived from floor plate, but rather a direct response to recombinant Shh peptides. To address this issue, and to determine accurately the response of neural explants to differing levels of Shh, peptides N1 and N3 were added directly to the medium. Even at the highest concentration (10 µg ml⁻¹), we were unable to induce *Shh* expression (N1, *n* = 6; N3, *n* = 15). In contrast, there was a dose-dependent increase in the induction of *Islet-1* expressing cells in response to both peptides, with a mean of 12–14 cells per explant at 100 ng ml⁻¹ to 130 cells per explant at 10 µg ml⁻¹ (Fig. 3). Double labelling with *Islet-1* and SC-1/BEN confirmed the motor-neuron identity of these cells (data not

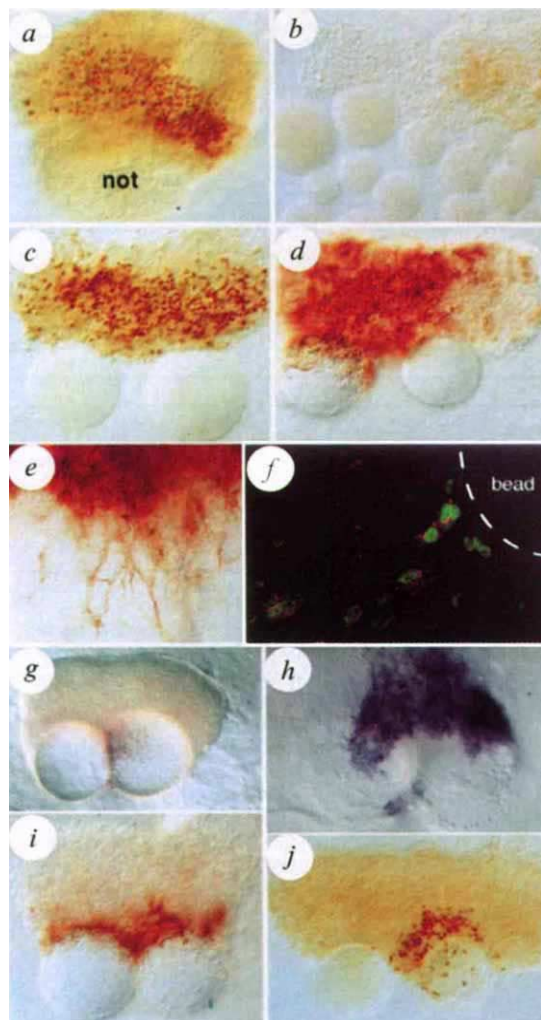


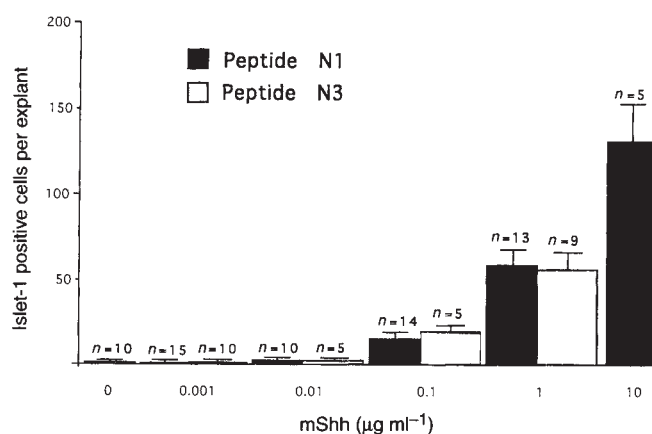
FIG. 2 *In vitro* induction of motor-neuron and floor-plate markers. *a*, Induction of motor neurons expressing Islet-1 by stage 6/7 chick notochord (not). *b*, Neural explants in contact with buffer-soaked nickel-agarose beads immunostained for Islet-1 expression. *c*, Induction of motor neurons expressing Islet-1 by N3-saturated beads. *d*, Induction of motor neurons expressing SC-1/BEN by N1-saturated beads. *e*, High magnification of axonal processes expressing SC-1/BEN in explants induced with N1-saturated beads. *f*, Coexpression of Islet-1 (fluorescein label, green) and of SC-1/BEN (rhodamine label, red) in motor neurons induced by N3-saturated beads. *g*, Neural explants in contact with buffer-soaked beads hybridized with *Shh* probe. *h*, Induction of *Shh* mRNA expressing cells by N3-saturated beads. *i, j*, Induction of SC-1/BEN (*i*) and Islet-1 (*j*) expression by beads loaded with subsaturating amounts of N3 (approximately 5-fold less than saturating levels).

shown). Taken together, the results indicate that peptides encompassing the mature 19K amino peptide are sufficient to induce both floor plate and motor neurons *in vitro*. However, the response is determined by the concentration of Shh ligand, and possibly also by the form in which the ligand is presented to the cell, whether bound to a surface or free in the medium.

To determine whether Shh production by the notochord is necessary for induction of motor neurons, we performed antibody blocking experiments using an affinity-purified rabbit polyclonal antiserum, which specifically recognizes the mature 19K peptide (Ab 80)^{2,30}. Control notochords incubated in Ringer's

solution (Fig. 4*a*) or pre-immune serum (Fig. 4*b*) gave a similar strong induction of Islet-1 expressing cells ranging from approximately 280 ($n=5$ out of 19) to over 400 cells per explant ($n=14$ out of 19). In contrast, in the presence of Ab 80, most explants gave fewer than 5 expressing cells (Fig. 4*d*; $n=13$ out of 22), with an upper limit of approximately 30 to 40 cells per explant (Fig. 4*c*; $n=6$ out of 22). Thus antibodies specific to the 19K peptide can markedly reduce, and in most cases completely abolish, induction of motor neurons. These results indicate that Shh signalling by the notochord is necessary for induction of motor neurons.

FIG. 3 Dose-dependent induction of motor neurons. Intermediate neural explants were cultured within collagen matrices in media supplemented with various amounts of Shh peptides (N1 and N3). Control explants were cultured in non-supplemented media. Each increase in the concentration of either peptide above 10 ng ml^{-1} resulted in the induction of a significantly greater number of motor neurons (N1, $X_{0.01} = X_{0.1}$, $P < 0.02$; $X_{0.1} = X_1$, $P < 0.001$; $X_1 = X_{10}$, $P < 0.005$; N3, $X_{0.01} = X_{0.1}$, $P < 0.02$; $X_{0.1} = X_1$, $P < 0.02$). At each specific concentration, no significant difference was observed in the number of motor neurons induced by each peptide ($X_{N1} = X_{N3}$; $P > 0.5$). Standard errors are indicated, as are the numbers (n) of explants analysed.



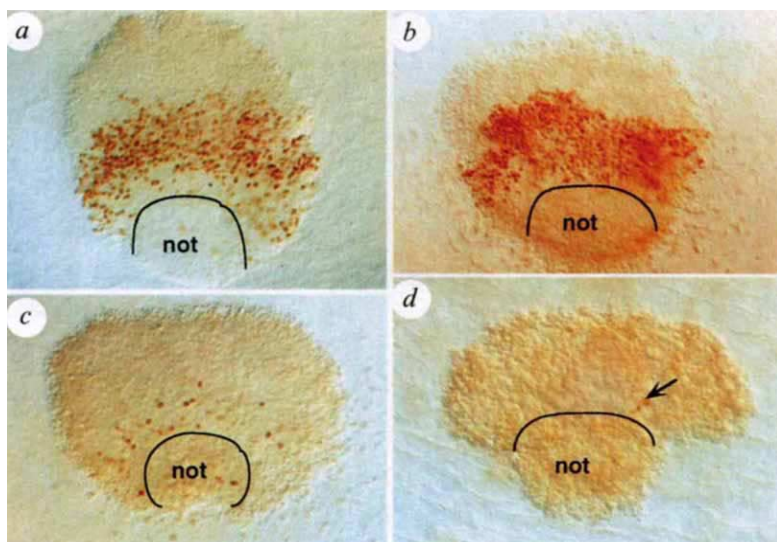


FIG. 4 Antibody-mediated blocking of motor-neuron induction by notochord. **a, b**, Examples of Islet-1 induction in neural plate explants cultured in contact with control notochords. Notochord in (**a**) was pre-incubated in Ringer's solution. Notochord in (**b**) was pre-incubated in pre-immune serum and cultured in the presence of pre-immune serum. **c, d**, Examples of Islet-1 induction in explants cultured in contact with notochords, in the presence of an affinity-purified rabbit polyclonal antiserum specific to the 19K N-terminal Shh peptide (Ab 80)²; arrow points to 2 Islet-1 expressing cells. METHODS. Notochords from stage 6 to 7 chick embryos²¹ were incubated for 3 h at 37 °C in a 5% CO₂ atmosphere in Ringer's solution, or Ringer's supplemented with either 50 µg ml⁻¹ of pre-immune serum or an affinity-purified antibody raised against the amino peptide of Shh (Ab 80)². Pre-immune serum (from the rabbit used to generate the Ab 80 antibody) was purified on a protein A-Sepharose CL-4B column (Pharmacia), eluted in 150 mM glycine (pH 2.5), neutralized, then dialysed against PBS. Affinity purification of Ab 80 is described elsewhere². Tissue recombinants were prepared and cultured as described in Fig. 1. Culture media for the experimental tissue recombinants was supplemented with 5 µg ml⁻¹ of Ab 80 and for the control notochords with 5 µg ml⁻¹ of pre-immune serum.

In summary, our results demonstrate that the active signalling component of Shh is the amino 19K peptide. A similar conclusion has recently been reached with respect to its *Drosophila* counterpart Hh^{20,27}. This is both necessary and sufficient for the induction of distinct ventral cell types *in vitro*. Thus the 19K peptide appears to act as a morphogen. Whether the different responses depend on the actual concentration of the factor itself or the method of presentation to the receiving cell is not yet clear. The demonstration that Shh can induce uncommitted neural progenitors to form motor neurons may have clinical significance. Moreover, the widespread expression of Shh in prechordal plate mesoderm, notochord and the ventral midline of the CNS along the entire rostral-caudal length of the CNS³⁰ raises the possibility that Shh may induce other ventral cell types at different axial levels. For example, it has recently been demonstrated that dopaminergic neurons, which arise in the ventral midbrain, are induced in response to a signal derived from floor plate²⁸. In addition, Shh has been implicated in patterning structures outside the CNS, most notably along the anterior-posterior axis of the limb¹⁸. It is not yet known whether recombinant Shh peptide is able to mirror the dose-dependent effects of ZPA signalling. Finally, there are obvious similarities with the role of *Drosophila* Hh. However, *Drosophila*, Hh appears to act indirectly, by regulating the expression of a second signal encoded by the *TGF-β* superfamily member *decapentaplegic*^{11,14,15,17}. We have no evidence for a second signal regulated by Shh in early neural tissue, however, suggesting that Shh may act directly to pattern the vertebrate CNS. □

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- Smith, J. C. *Curr. Biol.* **3**, 582–585 (1993).
- Bumcrot, D. A., Takada, R. & McMahon, A. P. *Molec. cell. Biol.* **15**, 2294–2303 (1995).
- Echelard, Y. et al. *Cell* **75**, 1417–1430 (1993).
- Krauss, S., Concordet, J.-P. & Ingham, P. W. *Cell* **75**, 1431–1444 (1993).
- Roelink, H. et al. *Cell* **76**, 761–775 (1994).
- Fietz, M. J. et al. *Development (Suppl.)* **120**, 43–51 (1994).
- Mohler, J. *Genetics* **120**, 1061–1072 (1988).
- Lee, J. L., von Kessler, D. P., Parks, S. & Beachy, P. *Cell* **71**, 33–50 (1992).
- Ingham, P. W. *Nature* **366**, 560–562 (1993).
- Heberlein, U., Wolff, T. & Rubin, G. M. *Cell* **75**, 913–926 (1993).
- Ma, C., Zhou, Y., Beachy, P. A. & Moses, K. *Cell* **75**, 927–938 (1993).
- Tabata, T. & Kornberg, T. B. *Cell* **76**, 89–102 (1994).
- Heemskerk, J. & DiNardo, S. *Cell* **76**, 449–460 (1994).
- Basler, K. & Struhl, G. *Nature* **368**, 208–214 (1994).
- Diaz-Benjumea, F. J., Cohen, B. & Cohen, S. M. *Nature* **372**, 175–179 (1994).
- Lee, J. J. et al. *Science* **266**, 1528–1537 (1994).
- Ingham, P. W. & Fietz, M. J. *Curr. Biol.* **5**(4), 432–441 (1995).
- Riddle, R. D., Johnson, R. L., Laufer, E. & Tabin, C. *Cell* **75**, 1401–1416 (1993).
- Chang, D. T. et al. *Development* **120**, 3339–3353 (1994).
- Porter, J. A. et al. *Nature* **374**, 363–366 (1995).
- Hamburger, V. & Hamilton, H. L. *J. Morph.* **88**, 49–92 (1951).
- Tsuchida, T. et al. *Cell* **79**, 957–970 (1995).
- Tanaka, H. & Obata, K. *Dev. Biol.* **106**, 26–37 (1984).
- Yamada, T., Placzek, M., Tanaka, H., Dodd, J. & Jessell, T. M. *Cell* **64**, 635–647 (1991).

- Thor, S., Ericson, J., Brännström, T. & Edlund, T. *Neuron* **7**, 881–889 (1991).
- Yamada, T., Pfaff, S. L., Edlund, T. & Jessell, T. M. *Cell* **73**, 673–686 (1993).
- Fietz, M. J., Jacinto, A., Taylor, A. M., Alexandre, C. & Ingham, P. W. *Curr. Biol.* (in the press).
- Hynes, M., Poulsen, K., Tessier-Lavigne, M. & Rosenthal, A. *Cell* **80**, 95–101 (1995).
- Dubendorff, J. W. & Studier, F. W. *J. molec. Biol.* **219**, 45–59 (1991).
- Martí, E., Takada, R., Bumcrot, D. A., Sasaki, H. & McMahon, A. P. *Development* (in the press).

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A critical period for long-term potentiation at thalamocortical synapses

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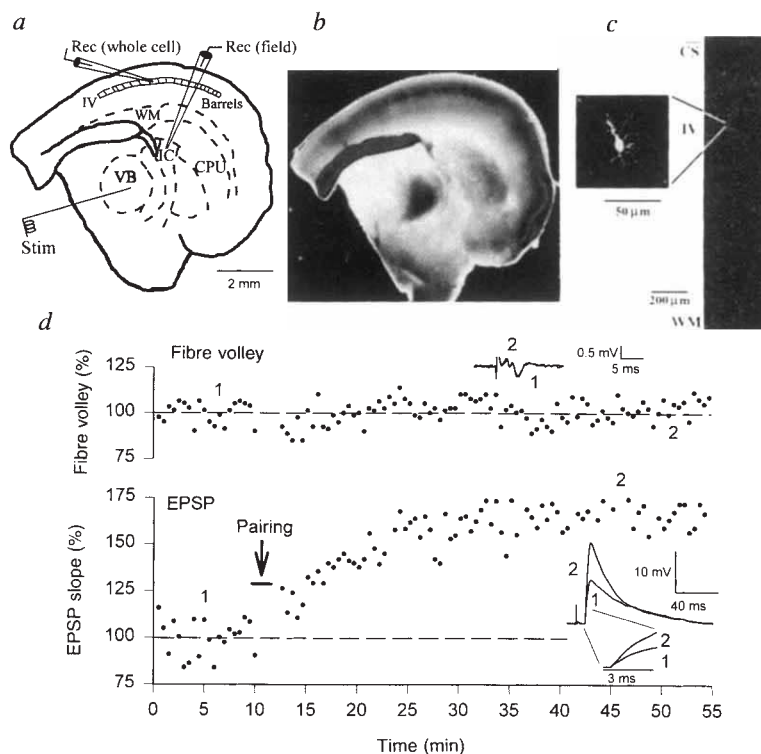
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IN mammalian development, the refinement of topographical projections from the thalamus to the cortex is thought to arise through an activity-dependent process in which thalamic axons compete for cortical targets^{1,2}. In support of this view, if activity is altered during a critical period in early development, normal connectivity is disrupted^{1,2}. It has been proposed that synaptic connections are strengthened during development by correlated pre- and postsynaptic activity^{3,4}, and a likely mechanism for this process would be *N*-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP)^{5,6}. However, the evidence that LTP is involved in normal development remains inconclusive. We have examined LTP in the thalamocortical synapses that form whisker barrels in rat somatosensory cortex (S1). We report here that the period during which LTP can be induced matches closely the critical period during which the barrels can be modified by sensory perturbations. Moreover, the loss of susceptibility to LTP with age is accompanied by a decrease in NMDA receptor-mediated synaptic currents. These findings provide compelling evidence that LTP is important for the development of cortical circuitry.

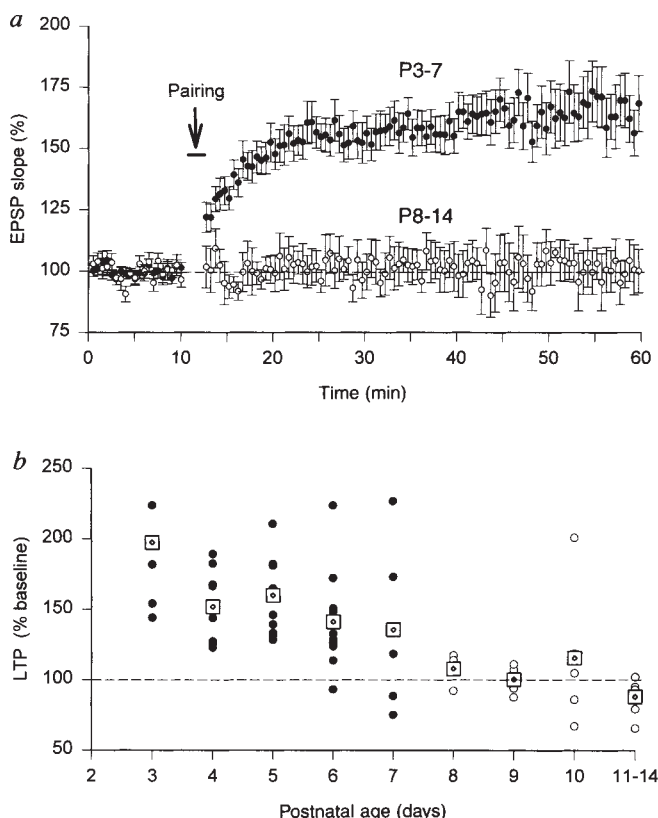
The barrels of S1 cortex are a somatotopic representation of sensory vibrissae on the rodent's muzzle which are formed by aggregates of ventrobasal thalamic (VB) afferents that innervate discrete clusters of layer IV cells^{7,8}. Although the development of the gross morphology of barrels does not appear to require

FIG. 1 Long-term potentiation (LTP) in layer IV cells of a thalamocortical slice preparation. *a*, Diagram of the preparation. Stimulating electrodes (Stim) are placed directly in the ventrobasal nucleus of the thalamus (VB) and monosynaptic responses are recorded from layer IV cells (Rec, whole cell). CPU, caudate/putamen; IC, internal capsule; WM, white matter. *b*, Slice preparation (P9) in the recording chamber, illustrating that transillumination reveals identifiable layer IV barrels and VB. *c*, An example of a fluorescent Nissl-stained section (P5) with a Lucifer yellow filled layer IV cell (CS, cortical surface). *d*, Example of LTP from P3 slice showing the slow growth of the potentiation in the absence of any change in the simultaneously recorded fibre volley from the internal capsule. Insets show example data traces taken at the times indicated by the numbers in the graph. Note that expansion of the traces demonstrates a clear increase in the initial slope of the e.p.s.ps.

METHODS. Slices from pups of timed-pregnant Sprague-Dawley rats (P0 is defined as the first 24 h after birth) were prepared as described¹². Recording techniques, data acquisition and data analysis were as described²⁹. Afferent stimulation was maintained at 0.033 Hz. The composition of the extracellular perfusing solution was 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃ and 11 mM glucose. The whole-cell recording pipette solution contained 117.5 mM caesium gluconate, 17.5 mM CsCl, 8 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP and 0.3 mM GTP (pH = 7.2, 280–290 mosmol). Cells were studied only if upon break-in they generated overshooting action potentials, had input resistances >300 M Ω , and synaptic responses were stable with no signs of drift for >10 min before LTP induction. Only one cell per slice was studied. For voltage-clamp experiments the fast peak of the excitatory postsynaptic current (e.p.s.c.) was measured. In all experiments, a fibre volley was simultaneously recorded from the internal capsule and the experiment was discarded if its amplitude increased. Data in the text and in the summary graphs are presented as mean $\pm$ s.e.m. except where noted.



The mean percentage change in e.p.s.p./e.p.s.c. magnitude was calculated by averaging over a 10 min window taken 30–40 min after the LTP induction protocol and comparing this value to the 10 min baseline period. Slices with Lucifer yellow CH (1%) filled cells were counterstained with the fluorescent Nissl stain bisbenzamide (2.5 μ g ml⁻¹) to identify laminae.



postnatal cortical activity⁹, manipulations of the sensory periphery during the critical period cause a profound rearrangement of the normal somatotopic patterning of VB afferents¹⁰, an effect that is activity dependent¹¹. Using a novel thalamocortical slice preparation¹² (Fig. 1a), we have examined the occurrence and properties of LTP at the synapse between VB afferents and layer IV cells. Transillumination of the slice (Fig. 1b) reveals cytoarchitectonic structures that clearly identify layer IV and VB, allowing for placement of microelectrodes under visual control. To confirm the location of layer IV in each slice, the characteristic profile of field excitatory postsynaptic potentials (e.p.s.ps) across laminae were recorded¹². In addition, several cells ($n=6$) were stained with Lucifer yellow, and all were found to be in layer IV (Fig. 1c). To ensure that monosynaptic e.p.s.ps were examined, recordings were rejected if there was any change in the latency of the e.p.s.p. when adjusting stimulation strength from threshold to saturation or during higher frequency (1 Hz) stimulation. Moreover, synaptic strength was assayed by measuring the very early initial slope of e.p.s.ps over the first 0.3–1.5 ms of the response.

Initially, we prepared slices from animals within one week of birth (postnatal day (P) 3–7) and attempted to elicit LTP by applying presynaptic stimulation (100 stimuli at 1 Hz to VB)

FIG. 2 LTP is limited to the critical period. *a*, Summary graphs of the effects on synaptic transmission of applying afferent stimulation (100 stimuli at 1 Hz) while the cell is depolarized (–10 to 0 mV) (pairing) in P3–7 slices ($n=43$) and P8–14 slices ($n=20$). *b*, A scatter diagram showing that the magnitude of LTP gradually decreases during the critical period. Each point represents a single cell which contributed to the graphs in *a*. For clarity, one P3 cell is not displayed (LTP magnitude = 282%). The squares show the mean LTP magnitude for that age.

during direct postsynaptic depolarization of layer IV cells (to between -10 mV and 0 mV), a protocol (termed 'pairing' in Figs 1–3) which ensures that the essential ingredients required for generation of LTP are provided. In P3–7 slices, this protocol elicited robust LTP of the monosynaptic e.p.s.p. in the absence of any change in the afferent fibre volley (Figs 1d and 2). Like other forms of cortical LTP¹³, the increase in synaptic strength developed slowly over 5–15 min before stabilizing for the duration of the recording. Consistent with the path length of axons from VB to S1, the latency of the e.p.s.ps was long (10.3 ± 0.6 ms, $n = 14$) and did not change following LTP (Fig. 1d).

After the first postnatal week, even severe sensory manipulations do not disrupt layer IV barrel field topography^{10,11}. To determine whether LTP at layer IV thalamocortical synapses is limited to the critical period, we examined the ability to generate LTP in slices prepared from older animals (P8–14). Although robust LTP could be generated in slices up to P7 ($158 \pm 6\%$, $n = 43$), thereafter it was extremely difficult to elicit any LTP ($102 \pm 6\%$, $n = 20$) (Fig. 2a). From P3 to P7, the magnitude of LTP gradually decreased (Fig. 2b) such that, by P8, LTP (greater than 120%) was observed in only 1 of 20 cells. The input resistance of layer IV cells also decreased with development (P3–7, 844 ± 68 M Ω , $n = 43$; P8–14, 420 ± 23 M Ω , $n = 20$, $P < 0.01$). However, this cannot account for the inability to elicit LTP in older slices because all cells were held near the e.p.s.p. reversal potential during the LTP induction protocol.

To understand the mechanisms responsible for the loss of LTP it was first necessary to define its basic properties in young slices (P3–6). We initially tested whether postsynaptic depolarization was in fact required for eliciting LTP, and found that voltage clamping cells at -95 mV prevented LTP ($96 \pm 9\%$, $n = 5$) (Fig. 3a). Depolarizing cells in the absence of afferent stimulation caused a small increase in synaptic strength (Fig. 3b) ($116 \pm 6\%$, $n = 18$) which was clearly much less ($P < 0.001$) than that elicited by the standard LTP induction protocol. To determine whether LTP requires a rise in postsynaptic calcium concentration, cells

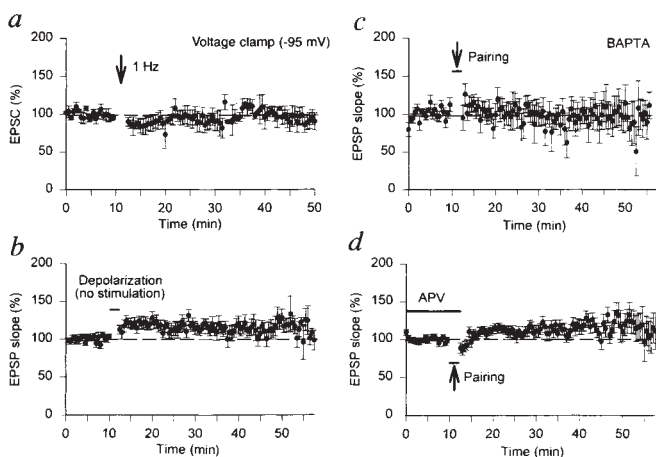


FIG. 3 Properties of LTP induction in P3–6 slices. **a**, Voltage clamping cells at -95 mV ($n = 5$) during 1 Hz stimulation (100 s) prevented the generation of LTP. **b**, Depolarizing cells ($n = 18$) (-10 to 0 mV for 100 s) in the absence of afferent stimulation did not elicit LTP. **c**, Loading cells ($n = 10$) with BAPTA (10 mM in the pipette solution) prevented the LTP normally elicited by combining afferent stimulation with postsynaptic depolarization ('pairing'). **d**, Bath applying the NMDA receptor antagonist D-APV (50 μ M; $n = 25$) prevented LTP. LTP is defined as an increase in synaptic strength $>120\%$. The small potentiation elicited by depolarization alone (**b**) or in the presence of APV (**d**) may be due to activation of voltage-dependent Ca^{2+} channels as it was blocked by hyperpolarization (**a**) or BAPTA (**c**).

METHODS. For all experiments, control cells (included in Fig. 2) were interleaved with experimental cells. BAPTA (10 mM) replaced caesium gluconate in the pipette solution to maintain osmolality.

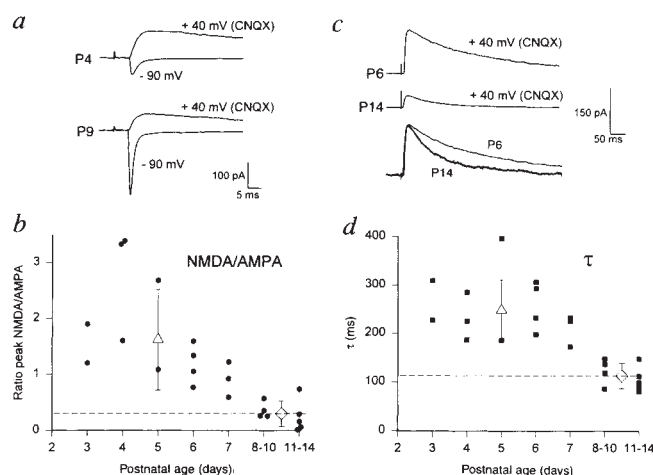


FIG. 4 NMDA receptor-mediated synaptic currents decrease with age. **a**, Sample e.p.s.cs from P4 and P9 slices recorded at -90 mV to measure AMPA receptor-mediated currents and at $+40$ mV in the presence of the non-NMDA receptor antagonist CNQX (10 μ M) and picrotoxin (100 μ M) to measure NMDA receptor-mediated currents. **b**, The ratio of peak NMDA receptor-mediated synaptic currents to AMPA receptor-mediated synaptic currents as a function of age. The open symbols indicate the mean $\pm$ s.d. for P3–7 and P8–14 cells. Note that all P3–7 cells had a ratio larger than the mean (broken line) for P8–14 cells. **c**, Sample NMDA receptor-mediated e.p.s.cs from P6 and P14 cells. Below, the peak of the P14 e.p.s.c. was aligned with that of the P6 e.p.s.c. to illustrate the difference in the decays. **d**, The time constant of decay of NMDA receptor e.p.s.cs as a function of age. Open symbols are as in **b**. Note that τ (time constant of decay; see Methods) for all P3–7 cells was larger than the mean (broken line) for P8–14 cells.

METHODS. E.p.s.cs were recorded at -90 mV for at least 10 min and then, during continued afferent stimulation, CNQX (10 μ M) and picrotoxin (100 μ M) were bath applied and the cell depolarized to $+40$ mV. The peak AMPA receptor- or NMDA receptor-mediated synaptic current was obtained by averaging together 10–20 consecutive e.p.s.cs at either holding potential. τ was calculated as the time it took the current to decay to $1/e$ of the peak current. In 24 of 25 cells, an NMDA receptor-mediated e.p.s.c. remained after application of CNQX, providing further evidence that monosynaptic responses were being recorded.

were loaded with the calcium chelator BAPTA (10 mM), which completely blocked the LTP normally elicited by stimulating afferents during postsynaptic depolarization (Fig. 3c) ($97 \pm 8\%$, $n = 10$). The requirements for afferent stimulation, postsynaptic depolarization and a rise in postsynaptic calcium suggested that synaptic activation of NMDA receptors is critically important for generating LTP. Consistent with this proposal, D-APV (50 μ M) strongly inhibited LTP (Fig. 3d) ($116 \pm 5\%$, $n = 25$).

Previous work has suggested that the properties of synaptic NMDA receptors change early during development^{14,15}, a change that in visual cortex¹⁵ occurs during a comparable period of development to that examined here. To determine if similar changes occur in layer IV thalamocortical synapses in S1, we examined pharmacologically isolated NMDA receptor-mediated synaptic currents. There was a profound decrease in the relative contribution of NMDA receptors to synaptic currents as a function of age (Fig. 4a, b), such that the ratio of currents mediated by NMDA receptors to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors decreased by over fivefold from 1.62 ± 0.9 (mean $\pm$ s.d.) for P3–7 slices ($n = 14$) to 0.30 ± 0.2 for P8–14 slices ($n = 9$; $P < 0.0001$). This change could be due either to a relative decrease in synaptic NMDA receptors or a relative increase in synaptic AMPA receptors. However, the time course of NMDA receptor synaptic currents also decreased dramatically with age (Fig. 4c, d). Their estimated time constant of decay decreased from 248 ± 62 ms (mean $\pm$ s.d.) for P3–7 cells ($n = 14$) to 113 ± 26 ms for P8–14 cells ($n = 9$, $P < 0.0001$). Thus,

independent of any change in the relative proportion of NMDA and AMPA receptors at S1 thalamocortical synapses, the properties of synaptic NMDA receptors have changed in a manner which would make it more difficult to elicit LTP.

Theoretical work^{3,4,16,17} suggests that hebbian synaptic modifications are critical for the precise patterning of neuronal connectivity during development. However, experimental evidence in support of this hypothesis has been limited because it is difficult to find manipulations that block such modifications (e.g. LTP) without affecting normal neural activity (see ref. 18). We report here that, at the thalamocortical synapses which form the barrels of rodent S1, LTP is limited to the first postnatal week and essentially cannot be generated thereafter, a time period which closely matches the critical period for the topographic reorganization of VB afferents by sensory experience. Although this strong correlation does not prove causality, it provides compelling evidence that NMDA receptor-dependent LTP is critical for the rearrangement of cortical maps induced by sensory experience. Consistent with this hypothesis is earlier work which demonstrated developmental changes in LTP at unidentified synapses in visual cortex¹⁹ and the occurrence of LTP at developing retinogeniculate synapses²⁰. Unlike layer IV cells that are directly activated by thalamic afferents, the peripherally evoked responses of cells in infra- and supragranular layers can be modified by sensory perturbations after the critical period^{21,22}. This may be attributable to the LTP which can be generated at intracortical connections in older animals¹³.

During the course of development, the molecular composition of NMDA receptors can change^{23,24} and thereby influence NMDA receptor inactivation kinetics^{25,26}. Because LTP is thought to require an increase in intracellular calcium concentration beyond some critical threshold²⁷, changes in NMDA receptors that decrease the time course and relative contribution of NMDA receptor-mediated synaptic currents provide a mechanism for the difficulty in eliciting LTP at thalamocortical synapses in S1 after the critical period. Changes in local inhibitory

circuits may also influence evoked NMDA receptor currents²⁸, although these cannot account for our results because inhibition was blocked when recording NMDA receptor-mediated synaptic currents. In visual cortex¹⁵ and superior colliculus¹⁴, the change in NMDA receptor properties appears to occur later in development than in S1, suggesting that the exact timing of critical periods in different brain areas may be dependent on the local molecular regulation of NMDA receptors. □

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- Goodman, C. S. & Shatz, C. J. *Cell* **72**, 77–98 (1993).
- Shatz, C. J. *Neuron* **5**, 745–756 (1990).
- Hebb, D. O. *The Organization of Behaviour* (Wiley, New York, 1949).
- Stent, G. S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 997–1001 (1973).
- Collingridge, G. L. & Singer, W. *Trends Neurosci.* **11**, 290–296 (1990).
- Kandel, E. R. & O'Dell, T. J. *Science* **258**, 243–245 (1992).
- Woolsey, T. A. & Van der Loos, H. *Brain Res.* **1**, 205–242 (1970).
- Killackey, H. P. & Leshin, S. *Brain Res.* **86**, 469–472 (1975).
- Chiala, N. L., Fish, S. E., Bauer, W. R., Bennett-Clarke, C. A. & Rhoades, R. W. *Dev. Brain Res.* **66**, 244–250 (1992).
- Woolsey, T. A. & Wann, J. R. *J. comp. Neurol.* **170**, 53–66 (1976).
- Schlaggar, B. L., Fox, K. & O'Leary, D. D. *Nature* **364**, 623–626 (1993).
- Agmon, A. & Connors, B. W. *Neuroscience* **41**, 365–379 (1991).
- Bear, M. F. & Kirkwood, A. *Curr. Opin. Neurobiol.* **3**, 197–202 (1993).
- Hestrin, S. *Nature* **357**, 686–689 (1992).
- Carmignoto, G. & Vicini, S. *Science* **258**, 1007–1011 (1992).
- Bienenstock, E. L., Cooper, L. N. & Munro, P. W. *J. Neurosci.* **2**, 32–48 (1982).
- Miller, K. D., Keller, J. B. & Stryker, M. P. *Science* **245**, 605–615 (1989).
- Fox, K. & Daw, N. W. *Trends Neurosci.* **16**, 116–122 (1993).
- Komatsu, Y., Fujii, K., Maeda, J., Sakaguchi, H. & Toyama, K. *J. Neurophysiol.* **59**, 124–141 (1988).
- Mooney, R., Madison, D. V. & Shatz, C. J. *Neuron* **10**, 815–825 (1993).
- Fox, K. *J. Neurosci.* **12**, 1826–1838 (1992).
- Diamond, M. E., Huang, W. & Ebner, F. F. *Science* **265**, 1885–1888 (1994).
- Watanabe, M., Inoue, Y., Sakimura, K. & Mishina, M. *Neuroreports* **3**, 1138–1140 (1992).
- Williams, K., Russel, S. L., Shen, Y. M. & Molinoff, P. B. *Neuron* **10**, 267–278 (1993).
- Monyer, H. et al. *Science* **256**, 1217–1221 (1992).
- Kutsuwada, T. et al. *Nature* **358**, 36–41 (1992).
- Malenka, R. C. & Nicoll, R. A. *Trends Neurosci.* **16**, 521–527 (1993).
- Agmon, A. & O'Dowd, D. K. *J. Neurophysiol.* **68**, 345–349 (1992).
- Mulkey, R. M. & Malenka, R. C. *Neuron* **9**, 967–975 (1992).

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Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience

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LONG-TERM potentiation (LTP) is a lasting enhancement of excitatory synaptic transmission that follows specific patterns of electrical stimulation¹. Although the mechanism of LTP has been intensively studied, particularly in the hippocampus, its significance for normal brain function remains unproven. It has been proposed that LTP-like mechanisms may contribute to naturally occurring, experience-dependent synaptic modifications in the visual cortex^{2–8}. The formation of normal binocular connections within the visual cortex requires simultaneous input from both eyes during a postnatal critical period^{9–12} that can be delayed by rearing animals in complete darkness^{13,14}. To explore the role of LTP in this experience-dependent maturation process, we induced LTP in visual cortical slices taken at different ages from light-reared and dark-reared rats. Susceptibility to LTP coincides with the critical period and, like the critical period, can be prolonged by rearing

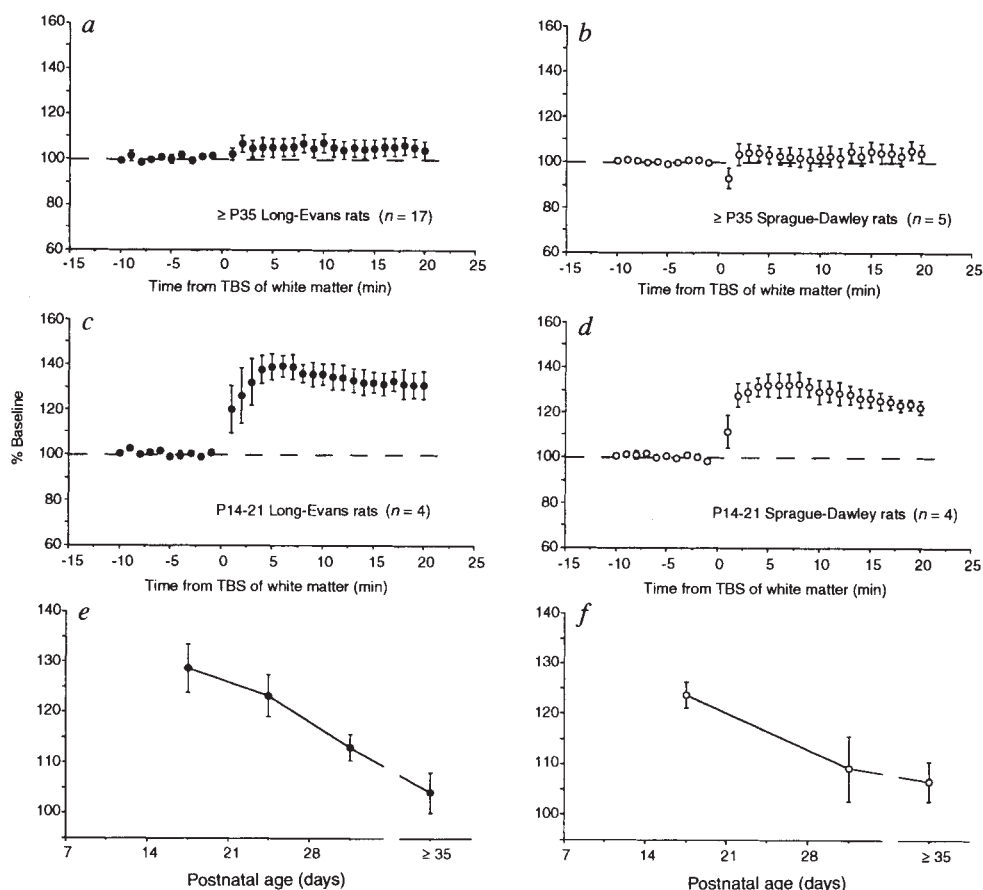
animals in darkness. These findings support the hypothesis that LTP reflects a normal mechanism of experience-dependent synaptic modification in the developing mammalian brain.

For many years the visual cortex has been used as a model to study experience-dependent synaptic plasticity. Modifications can be elicited *in vivo* with simple manipulations of visual experience, such as monocular deprivation, and these modifications have clear behavioural consequences, such as blindness of the deprived eye¹⁵. Although carnivores and primates have traditionally been used for studies of experience-dependent visual cortical development, the same principles apply to rodents¹². In rat visual cortex, for example, visual response properties mature rapidly from postnatal day (P) 18, when the optics clear, to P45. As in other species, dark-rearing from birth postpones this maturation. Moreover, response properties in the small binocular region of rat visual cortex can be altered by monocular deprivation during a critical period. Susceptibility of binocular connections to monocular deprivation is greatest when deprivation is begun at approximately three weeks of age but declines rapidly, such that deprivation initiated at 5 weeks of age has little effect^{12,16}.

Various forms of activity-dependent modifications have been described by studying synaptic plasticity in the visual cortex *in vitro*^{2–6,17}. The common approach has been to record intracellular or population excitatory postsynaptic potentials in layer III that are evoked by electrical stimulation at the border of the white matter and layer VI. In the absence of treatments to reduce inhibition, conditioning stimulation of the white matter fails to produce robust LTP in layer III in slices of visual cortex prepared from normal adult ($\geq$ P35) rats^{3,7,17}. However, it has been reported that LTP may be elicited using this stimulation-

FIG. 1 Developmental decline in LTP in rat visual cortex. *a, b*, Average effect of TBS of the white matter on the field potential amplitude in layer III of visual cortical slices from *a*, pigmented, and *b*, albino rats aged $\geq P35$. *c, d*, Average effect of TBS of the white matter on the field potential amplitude in layer III of visual cortical slices from *c*, pigmented, and *d*, albino rats aged 2–3 weeks postnatal (P14–21). *e, f*, LTP evoked in layer III by TBS of white matter plotted as a function of age. Each point is the normalized field potential amplitude 20 min following TBS averaged across rats ($n \geq 4$) in each age group (by 20 min the measured LTP in visual cortex is stable for at least 1 h). For rats younger than P35, the data collected in the given postnatal week were collapsed and averaged to yield the single points shown in the graph. The range of ages in the $P \geq 35$ group was 35–49. Data in *a–d* are expressed as follows. For each slice exhibiting a stable baseline, the field potential amplitudes were calculated as percentages of the baseline average, and the timescale was converted to time from TBS. The time-matched, normalized data were averaged across slices to yield a single data set for each rat. These data were then averaged across rats to yield the values shown in the graph, expressed as the means $\pm$ s.e.m.

METHODS. Slices of visual cortex (400 μ m) were prepared as previously described⁶. The slices were maintained in an atmosphere of humidified 95% O_2 and 5% CO_2 , and superfused with 35 $^\circ$ C artificial cerebrospinal fluid (ACSF) at a rate of 1 ml min^{-1} . The ACSF was saturated with 95% O_2 and 5% CO_2 , and contained 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4 , 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM dextrose. Glass microelectrodes (1–2 M Ω) were filled with ACSF and positioned in layer III, approximately 400 μ m below the pial surface. Field potentials were evoked with pulses of 10–100 μ A amplitude lasting 0.2 ms delivered with a bipolar concentric stimulating electrode (outside diameter 200 μ m) placed either just below the border of the white-matter and layer VI, or in the middle of the cortical thickness. The amplitude of the maximum negative field



potential in layer III was used as a measure of the evoked population excitatory synaptic current. As discussed in more detail elsewhere⁷, changes in the amplitude of the maximum negative field potential reflect changes in the magnitude of a synaptic current sink, and correlate with changes in the initial slope of excitatory postsynaptic potentials recorded intracellularly in layer III neurons. In every experiment, a full input–output curve was generated, and baseline responses were obtained at 0.07–0.03 Hz with a stimulation intensity that yielded a half-maximal response. To induce LTP, 3 episodes of TBS were delivered at 0.1 Hz. TBS consisted of 10 to 13 stimulus trains delivered at 5–7 Hz; each train consisted of 4 pulses at 100 Hz.

recording configuration in immature animals^{18–20}, suggesting that visual cortical LTP, like experience-dependent plasticity, is more robust during a critical period. We investigated these observations, and then extended the analysis to animals that had been reared in complete darkness.

The results of the developmental study, performed using slices of visual cortex from both Long-Evans pigmented rats and Sprague-Dawley albino rats, are summarized in Fig. 1. In agreement with previous findings⁷, theta burst stimulation (TBS) of the white matter did not produce substantial LTP in layer III of visual cortex from rats older than P35 (Fig. 1*a, b*). In contrast, TBS produced a robust synaptic enhancement in slices from animals in their third postnatal week (P14–21) (Fig. 1*c, d*). Figure 2*a* shows an example of LTP in one experiment. The developmental decline in LTP, expressed as the percentage change from baseline 20 min after TBS, is plotted for both strains of rat in Fig. 1*e, f*. This change in LTP from 3 to 5 weeks of age closely parallels the developmental decline in the effects of monocular deprivation on binocular connections *in vivo*¹².

To investigate whether the developmental decline in LTP is altered by the rearing history of the animals, litters of rats were

placed in a dark room within a few days of birth until approximately 5 weeks of age. Slices from age-matched light-reared and dark-reared animals were studied simultaneously on two separate slice rigs. The experiments were performed in two series, depending on whether the experimenters knew the rearing history of the animals ($n = 19$ rats) or not ($n = 12$ rats). The results were virtually identical: the developmental decline in LTP evoked from the white matter was prevented by dark-rearing the animals.

Figure 3*a* shows the results from the 'blind' series of experiments. TBS of the white matter produced little change in the light-reared animals, but the age-matched dark-reared animals showed robust LTP (see Fig. 2*b*). The pooled data from both series of experiments are shown in Fig. 3*b*, and the data from each individual slice appear in a cumulative histogram²¹ in Fig. 3*c*. The light-reared and dark-reared distributions are significantly different at $P < 0.02$ (Kolmogorov–Smirnov test). We observed no obvious differences, other than LTP, in the extracellular synaptic responses of slices in dark-reared animals compared with light-reared animals (no significant differences in field potential amplitude at half-maximal stimulation intensity, width at half-amplitude, or time to peak).

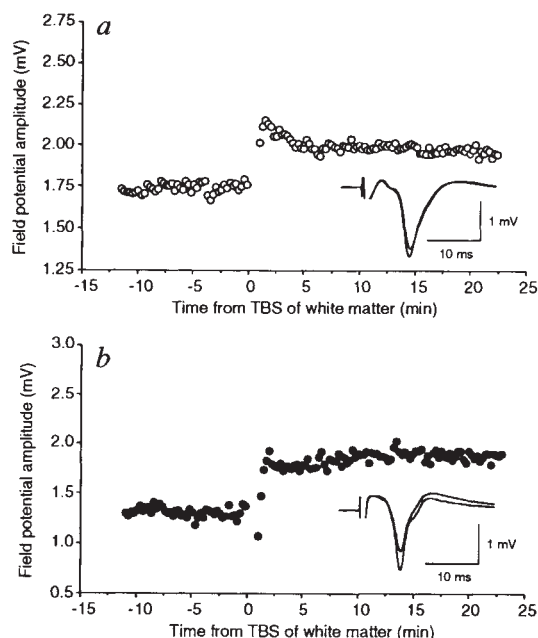
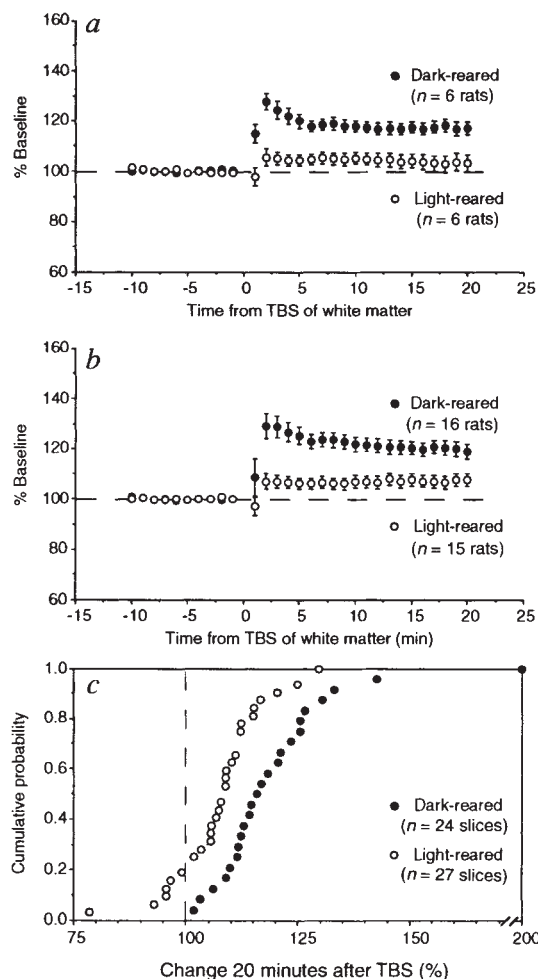


FIG. 2 Examples of LTP of the field potential evoked by white matter stimulation in visual cortical slices from a, a normal P16 rat, and b, a dark-reared P34 rat.



We conclude that white-matter-evoked LTP in layer III, and experience-dependent synaptic plasticity, are co-regulated in visual cortex by age and visual experience. Although it is not known precisely what is regulated, the results of additional experiments help to narrow the possibilities. We have previously shown that stimulation of the middle of cortex, corresponding roughly to layer IV, could yield LTP reliably in layer III of slices from adult visual cortex^{7,17}. We therefore investigated whether there are quantitative effects of age and dark-rearing on layer-IV-evoked LTP in layer III. In striking contrast to the effect of white-matter stimulation, however, we found that TBS of the middle cortical layers yields LTP of comparable magnitude and probability, regardless of age (in the range examined) or rearing history (Fig. 4). These data suggest that the developmental regulation of plasticity does not occur at the level of the biochemical machinery of the layer III neurons expressing the LTP.

These forms of LTP in visual cortex require *N*-methyl-D-aspartate (NMDA) receptor activation^{7,20}, and there is evidence for a developmental decline in NMDA receptor function in layer IV of visual cortex, which is prevented by dark-rearing^{22,23}. Because layer IV provides input to layer III, the LTP measured in layer III as a result of white-matter stimulation may be simply a reflection of a synaptic modification that actually occurs in layer IV neurons. However, dark-rearing has been shown to decrease, rather than increase, LTP of layer IV synaptic current sinks²⁴.

More work will be required to determine how LTP is developmentally regulated in visual cortex, but robust white-matter-

FIG. 3 Effect of dark-rearing on LTP evoked from the white matter in 4–6-week-old rat visual cortex. a, Comparison of LTP in visual cortical slices from 4–5-week-old rats reared in complete darkness with LTP in rats reared in a normal lighted environment. In this series, the experimenters were 'blind' to the rearing history of the animals until all analyses had been performed. Data are expressed as in Fig. 1a–d; the difference between groups at 20 min post-TBS is significant at $P < 0.02$ (*t*-test). b, The same as a, except that additional animals, not studied 'blind', are included in the averages. c, Cumulative histogram using all slices from dark-reared and light-reared animals. These distributions are significantly different at $P < 0.02$ (Kolmogorov–Smirnov test).

METHODS. Pregnant albino rats were purchased from Charles River laboratories, always timed so that two would give birth on approximately the same day. Within a week of giving birth, a mother and her pups were placed in a light-tight room while another was kept in a normal lighted laboratory environment (12 h light/dark cycle). To provide care for animals in the dark, or to remove them for experiments, they were illuminated with dim infrared light and viewed using infrared vision equipment. In the 'blind' series of experiments, one dark-reared and one light-reared animal was used each day, studied simultaneously on two slice rigs. For each slice, LTP was first attempted with middle layer stimulation, which other experiments had shown to be unaffected (on average) by rearing history or age (Fig. 4). If plasticity was observed then LTP was attempted from the white matter at a different location on the same slice; if no plasticity was observed, the slice was not studied further. We used this criterion to ensure that only high-quality slices were used in the 'blind' study. The only criterion used for the non-blind study, however, was a stable baseline. To construct cumulative histograms, the percentage change in the response 20 min after TBS was calculated for each slice, and these values were rank-ordered to show the fraction of cases showing changes of various magnitudes.

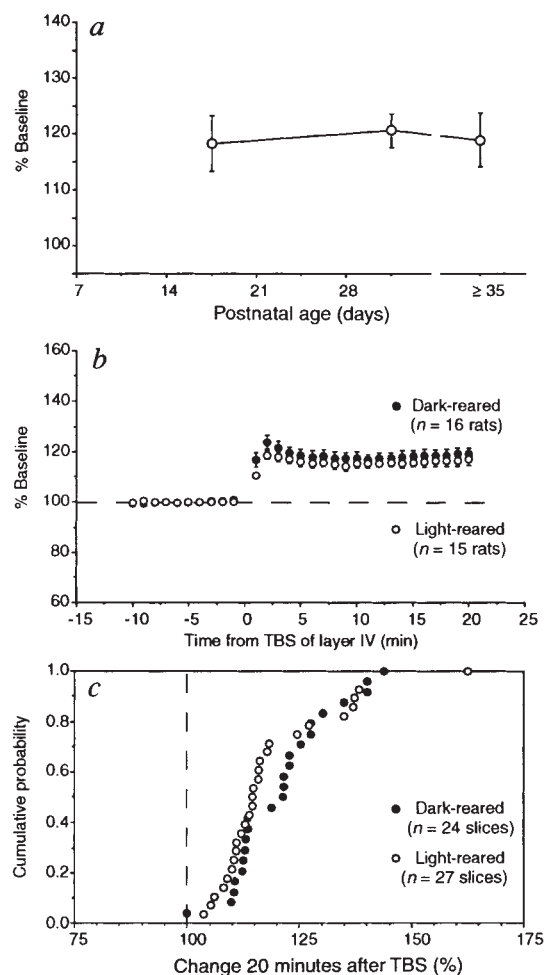


FIG. 4 Effect of age and visual experience on LTP evoked from the middle cortical layers. *a*, LTP evoked in layer III by TBS of the middle cortical layers plotted as a function of age. Data are calculated and expressed as in Fig. 1*e, f*. *b*, Comparison of LTP in visual cortical slices from 4–5-week-old rats reared in complete darkness with LTP in rats reared in a normal lighted environment. *c*, Cumulative histogram using all slices from age-matched dark-reared and light-reared animals. These distributions are not significantly different from one another.

evoked LTP can be restored in slices from adult animals if GABA_A-receptor-mediated inhibition is reduced^{3,7}. Inhibitory circuits may contribute to a 'plasticity gate' which controls the activity of middle-layer cells that must be recruited to induce LTP in layer III⁷. Our observations suggest that the gate is open early in postnatal life and closes during the critical period under the influence of experience. Closure of the hypothetical plasticity gate could result from maturation of excitatory or inhibitory synapses in the middle cortical layers; indeed, there is evidence for changes due to development and experience in both types of transmission^{25–30}.

Our experiments have shown that activity-dependent synaptic plasticity *in vitro*, like experience-dependent synaptic plasticity *in vivo*, is regulated by age and previous sensory experience. These findings are significant for three reasons. First, they support the relevance of LTP for understanding naturally occurring synaptic modifications in the mammalian brain. Second, they indicate that the stimulation history of the synapses *in vivo* leaves a trace that can be detected *in vitro*. Third, they suggest that the study of LTP in visual cortex may be useful for understanding the factors that regulate experience-dependent plasticity during critical periods of development. □

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- Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).
- Komatsu, Y., Toyama, K., Maeda, J. & Sakaguchi, H. *Neurosci. Lett.* **26**, 269–274 (1981).
- Artola, A. & Singer, W. *Nature* **330**, 649–652 (1987).
- Berry, R. L., Teyler, T. J. & Taizhen, H. *Brain Res.* **481**, 221–227 (1989).
- Kimura, F., Nishigori, A., Shirokawa, T. & Tsumoto, T. *J. Physiol., Lond.* **414**, 125–144 (1989).
- Bear, M. F., Press, W. A. & Connors, B. W. *J. Neurophysiol.* **67**, 1–11 (1992).
- Kirkwood, A. & Bear, M. F. *J. Neurosci.* **14**, 1634–1645 (1994).
- Frégnac, Y., Burke, J. P., Smith, D. & Friedlander, M. J. *J. Neurophysiol.* **71**, 1403–1421 (1994).
- Hubel, D. H. & Wiesel, T. N. *J. Physiol., Lond.* **206**, 419–436 (1970).
- Blakemore, C. & vanSluyters, R. C. *J. Physiol., Lond.* **237**, 195–216 (1974).
- Olson, C. R. & Freeman, R. D. *Expl Brain Res.* **39**, 17–21 (1980).
- Fagioli, M., Pizzorusso, T., Berardi, N., Domenici, L. & Maffei, L. *Vision Res.* **34**, 709–720 (1994).
- Cynader, M. & Mitchell, D. E. *J. Neurophysiol.* **43**, 1026–1039 (1980).
- Mower, G. D. *Dev Brain Res.* **58**, 151–158 (1991).
- Sherman, M. S. & Spear, P. D. *Physiol. Rev.* **62**, 738–855 (1982).
- Domenici, L., Berardi, N., Carmignoto, G., Vantini, G. & Maffei, L. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8811–8815 (1991).
- Kirkwood, A., Dudek, S. D., Gold, J. T., Aizenman, C. D. & Bear, M. F. *Science* **260**, 1518–1521 (1993).
- Komatsu, Y., Fujii, K., Maeda, J., Sakaguchi, H. & Toyama, K. *J. Neurophysiol.* **59**, 124–141 (1988).
- Perkins, A. T. & Teyler, T. J. *Brain Res.* **439**, 222–229 (1988).
- Kato, N., Artola, A. & Singer, W. *Dev Brain Res.* **60**, 43–50 (1991).
- Stevens, C. F., Tonegawa, S. & Wang, Y. *Curr. Biol.* **4**, 687–693 (1994).
- Fox, K., Daw, N., Sato, H. & Czeplita, D. *Nature* **350**, 342–344 (1991).
- Carmignoto, G. & Vicini, S. *Science* **258**, 1007–1011 (1992).
- Berry, R. L., Perkins, A. T. & Teyler, T. J. *Brain Res.* **628**, 99–104 (1993).
- Blue, M. E. & Parnavelas, J. G. *J. Neurocytol.* **12**, 697–712 (1983).
- Komatsu, Y. *Dev Brain Res.* **8**, 136–139 (1983).
- Bode-Gruel, K. M. & Singer, W. *Dev Brain Res.* **46**, 197–204 (1989).
- Luhmann, H. J. & Prince, D. A. *Dev Brain Res.* **54**, 287–290 (1990).
- Friauf, E. & Shatz, C. J. *J. Neurophysiol.* **66**, 2059–2071 (1991).
- Komatsu, Y. *J. Neurosci.* **14**, 6488–6499 (1994).

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Soluble antigen can cause enhanced apoptosis of germinal-centre B cells

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GERMINAL centres are dynamic microenvironments of B-lymphocyte differentiation, which develop in secondary lymphoid tissues during immune responses^{1–3}. Within germinal centres, activated B lymphocytes proliferate and point mutations are rapidly introduced into the genes encoding their immunoglobulin receptors^{4–10}. As a result, new specificities of B cells are created, including those with a heightened capacity to bind the immunizing antigen^{4–11}. Immunoglobulin gene mutation can also lead to reactivity to self antigens^{12–14}. It has been suggested that any newly formed self-reactive B cells are eliminated within the germinal centre in order to avoid autoimmunity^{15,16}. Here we present evidence that antigen-specific, high-affinity, germinal-centre B cells are rapidly killed by apoptosis *in situ* when they encounter soluble antigen. The effect seems to act directly on the B cells, rather than through helper T cells. Furthermore, the apoptosis is unique to germinal-centre cells, and is only incompletely impeded by constitutive expression of the proto-oncogene *bcl-2*. This phenomenon may reflect clonal deletion of self-reactive B cells within germinal centres.

A mouse model was developed to simulate a situation in which hypermutating, germinal-centre B cells would encounter soluble self-antigen, not complexed to antibody on the surface of follicu-

lar dendritic cells (FDC). C57BL/6 mice were immunized with the T-cell-dependent, hapten-carrier antigen (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to human serum albumin^{3,5-7,17,18} (HSA), and 14 days later, at the height of the immune response, the mice were injected with a large dose of soluble, deaggregated NP-HSA at various times before being killed. In contrast to the immunizing NP-HSA (which was precipitated in alum), the soluble NP-HSA has a low haptenation ratio (NP₁:HSA), which enables only mutated cells of higher affinity to bind it^{6,16,19}.

Apoptosis in the spleens of the mice was monitored histologically by the recently introduced TUNEL technique²⁰. Immunized mice which had not been injected with soluble NP-HSA showed a basal level of apoptosis (Figs 1*b* and 2) in the germinal centres. In contrast, immunized mice that received soluble NP-HSA at day 14, either 4 h or 6 h before being killed, showed a great increase in the number of apoptotic cells within the germinal centres (Figs 1*c* and 2). To establish the specificity of this phenomenon, immunized mice were injected with an identical dose of soluble bovine transferrin, an irrelevant antigen, 6 h before being killed. Such mice showed no significant rise in the number

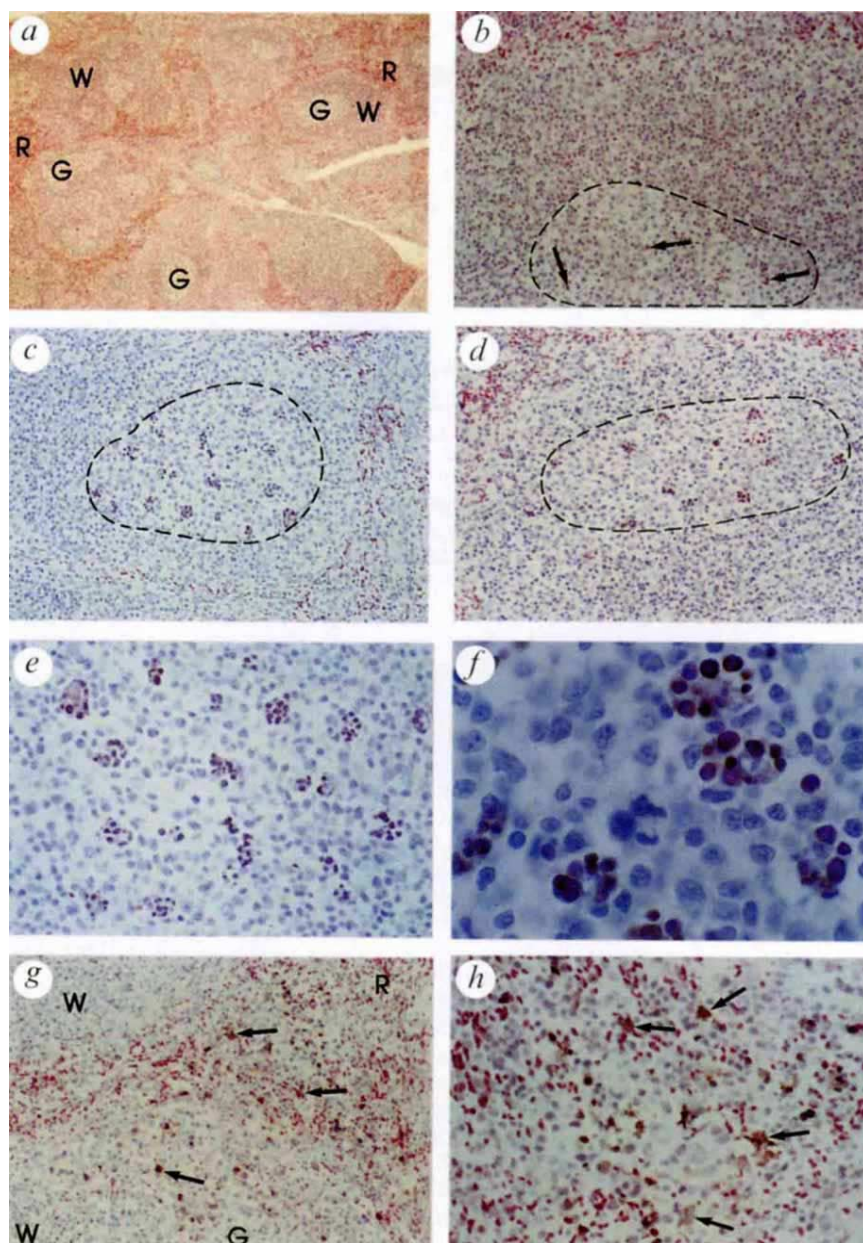
of apoptotic cells (Fig. 3), thus confirming that the apoptosis occurred in an antigen-specific way.

The apoptotic cells occurred in clusters (Fig. 1*c*). Frequently, apoptotic nuclei were clearly contained in tingible body macrophages. In other cases macrophages were not clearly apparent, raising the possibility that a group of clonally related cells had bound the soluble antigen and undergone apoptosis. The apoptosis occurred in a rapid wave, with a significant increase in the number of apoptotic cells being observed as early as 1 h after injection of soluble antigen (Fig. 2). By 12 h, the number of apoptotic cells was on the decline and both the germinal centres and the marginal zones contained macrophages containing highly fragmented dead cell debris (Fig. 1*g, h*). These macrophages were also detected in the neighbouring red pulp (Fig. 1*g, h*). By 24 h, the number of apoptotic cells in the germinal centres had declined to the original basal levels (Fig. 2).

The question of whether the soluble NP-HSA directly delivered a negative signal to the B cell, or whether it killed the B cells by depriving them of T-cell help, was addressed. Mice were immunized with NP-HSA precipitated in alum, and 14 days later

FIG. 1 Detection of apoptotic cells *in situ* with the TUNEL²⁰ technique, in which fragmented DNA strands within apoptotic cells are nick end-labelled with biotinylated poly (dU) introduced by terminal deoxynucleotidyl transferase, and then stained using avidin-conjugated peroxidase. Paraffin-embedded splenic sections (3 μ m thick) from various cohorts of mice described in the text were stained for apoptotic cells (brown), as described²⁰. Sections were counterstained with haematoxylin and eosin. Magnifications given are the original magnifications. *a*, The white pulp (W), red pulp (R) and germinal centres (G) could be identified. Germinal centre (GC) cells have their characteristic size and spacing ($\times 40$). *b*, GC (outlined) from a day 14 immunized mouse showing few apoptotic cells (arrowed) after injection of soluble NP₁-HSA ($\times 200$). In *c* and *d*, by contrast, extensive apoptosis is seen within the GC (outlined) 1 h (*c*), 4 h (*d*) and 6 h (not shown) after injection of soluble NP₁-HSA. In *c* and *d* and also in *e* and *f*, apoptotic cells occur in clusters and are uniformly distributed throughout the GC, suggesting that cells in both the dark zone and the light zone are killed by soluble antigen. *e*, $\times 400$; *f*, $\times 1,000$. *g, h*, By 12 h after injection of soluble NP₁-HSA, the GC, marginal zones, and follicular mantles contain macrophages (arrowed) which are filled with debris of apoptotic cells (brown). Such macrophages are also seen in the red-pulp at 12 h (not earlier), perhaps indicating migration. *g*, $\times 200$; *h*, $\times 400$.

METHODS. Briefly, C57BL/6 mice were immunized intraperitoneally (i.p.) with 5 mg soluble, deaggregated NP₁-HSA at either 1 h, 4 h, 6 h, 12 h or 24 h before being killed. Control mice received either no soluble injections or 5 mg of soluble bovine holotransferrin. The source of NP and HSA and the conjugation procedure used is described¹⁶. Transferrin (bovine holotransferrin) was purchased from Sigma.



they were injected with soluble, deaggregated forms of either NP-HSA, NP-MSA (mouse serum albumin) or HSA alone. The rationale here was that the soluble NP-MSA would only be recognized by B cells and not by helper T cells, as MSA-specific T cells should not exist in a mouse. In contrast, HSA would only be recognized by HSA-specific helper T cells, and not by NP-specific B cells. Therefore, if the soluble NP-HSA was acting directly on the B cells, both NP-HSA and NP-MSA should cause apoptosis, whereas HSA alone should not. However, if the soluble antigen was acting by depriving the B cells of T-cell help, then HSA alone should cause apoptosis, whereas NP-MSA should not. The results of these experiments showed that NP-MSA caused a similar level of apoptosis to NP-HSA, whereas HSA alone only caused a small increase, which presumably reflected the death of HSA-specific B cells (Fig. 3). This result, together with the rapid kinetics of the apoptosis, suggests that NP-HSA acts directly on the B cells.

We have previously shown that soluble NP₂-HSA injected 6 days after immunization with NP₁₆-HSA results in an impairment of memory-cell generation¹⁶. Through hapten-carrier studies, it was shown that this process occurred largely through an effect on the T cells. Although the nature of the experiments and assays used in the previous study is different, there seem to be two events censoring memory cell output in the germinal centre—one of rapid onset and acting directly on the B cell, as described in this study, and the other more gradual and acting by tolerizing the T cell, as described in the previous study.

To address the issue of whether the antigen-induced apoptosis could be due to complement-mediated lysis of antigen-specific germinal-centre B cells, we performed a preliminary experiment aimed at deplementing the mice with cobra venom factor. The mean number of apoptotic cells per germinal centre was

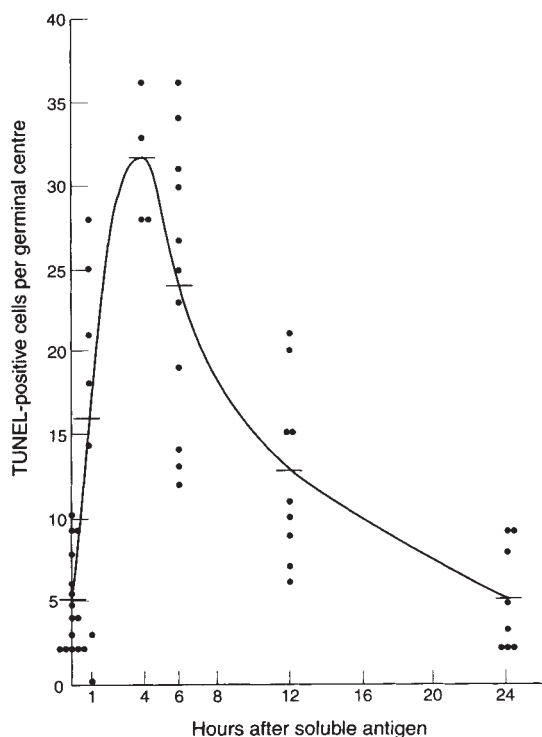


FIG. 2 Kinetics of apoptosis in germinal centres. As early as 1 h after injection of soluble antigen, there is a significant increase in the number of apoptotic cells within the follicles. Peak numbers of apoptotic cells occur 4–6 h after injection, and decline to near basal levels by 24 h. Each dot represents the mean number of apoptotic cells per follicle from an individual spleen. Large numbers of follicles were counted in each spleen. The arithmetic means are shown for each time point. The data are pooled from three independent experiments.

slightly, but not significantly, reduced from 17.6 ± 2.9 to 13.4 ± 2.0 . Although the possibility of immune complexes binding to Fc receptors and fixing complement cannot be ruled out from this one experiment, it seems unlikely, as any immune complexes formed would have been in antigen excess, believed to have immunostimulatory properties *in vivo*²¹.

The effect of the constitutive expression of the proto-oncogene *bcl-2* on this apoptosis was investigated. Bcl-2 prevents apoptosis in many different situations²² and its vital role in lymphoid development is evident in mice deficient for *bcl-2*, which lose all their lymphocytes shortly after birth^{23,24}. In addition, *bcl-2* has been shown to impede negative selection of B lymphocytes both in the bone marrow²⁵ and the periphery²⁶. Bcl-2 is not expressed in the germinal centre, apart from low levels at the apical light zone²⁷. It is thought to play a role in positive selection of memory cells leaving the germinal centre^{28,29}.

Transgenic mice from the *bcl-2*-22 line³⁰ which constitutively express the human *bcl-2* gene in B lymphocytes including those of germinal centres²⁹, and their littermate controls, were immunized with NP-HSA, and 14 days later, soluble NP₁-HSA was injected 6 h before killing the mice. Soluble antigen induced greatly enhanced apoptosis in a large fraction of the germinal centres in the littermate control mice (Fig. 4a). However, in the *bcl-2* transgenic mice, enhanced apoptosis could be detected in a smaller fraction of the germinal centres, perhaps indicating that a smaller proportion of these germinal centres were antigen-specific. The increase in apoptosis even within this subset of

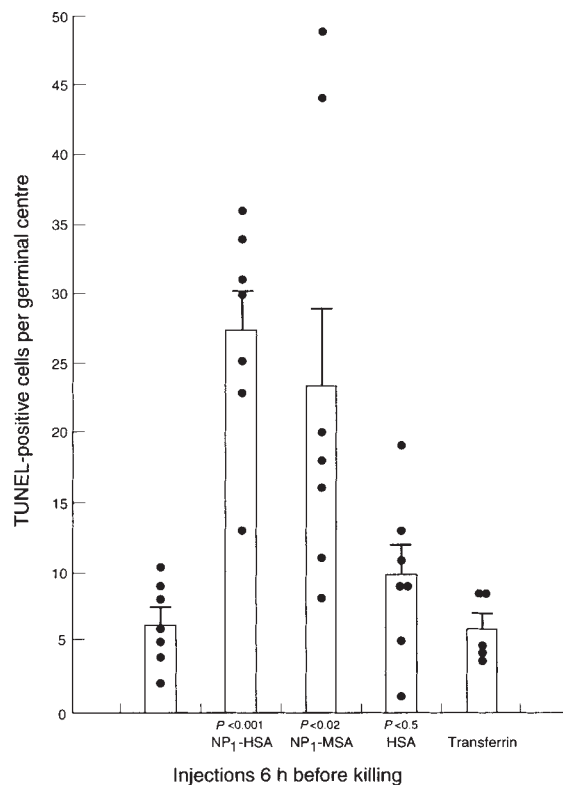


FIG. 3 Soluble antigen kills the B cells directly. Soluble injections of either NP₁-HSA or NP₁-MSA, 6 h before killing results in greatly enhanced apoptosis within the follicles. In contrast, soluble injection of HSA only gives a small increase in apoptosis. This suggests that apoptosis is mediated through NP, and is consistent with a direct effect on the B cells. The minor increase in apoptosis due to HSA may reflect the death of HSA-specific cells. Data are derived from two independent experiments in which seven mice were used in each group. Each dot represents one mouse. The arithmetic means ($\pm$ s.e.m) are shown as bars for each group. P values (Student's *t*-test) indicate significance of the differences of the means in the various groups compared to uninjected controls.

germinal centres in *bcl-2* mice, was moderately less than in controls (Fig. 4b). This indicates that *bcl-2* confers only incomplete protection from apoptosis caused by soluble antigen. Any difference between this result and those of Shokat *et al.*³¹ may be attributable to differences in the affinity of binding between NP-specific and HEL-specific cells.

Finally, to determine whether the observed cell death was unique to germinal centre cells, mice were injected with soluble NP₁-HSA eight days after immunization with NP-HSA and analysed as above. At this time point in this immune response, foci of extrafollicular cells forming anti-NP antibody are at maximal size³. The sections were double-stained to detect any TUNEL-positive cells in IgG1 foci (K.G.C.S., T. D. Hewitson, G.J.V.N. and D. M. Tarlinton, manuscript in preparation). A large number of cells in IgG1 foci were counted, and the number of apoptotic cells constituted only 2–3% of all cells in the foci of both the immune and tolerant spleens. Therefore, the enhanced apoptosis caused by the soluble antigen appears to be specific to germinal centres.

In conclusion, we have shown that soluble antigen causes the apoptosis of antigen-specific germinal centre B cells. This apoptosis exhibits very rapid kinetics and appears to act directly

on the B cells. It is only partially impeded by constitutive expression of *bcl-2*, and seems unique to germinal centre cells. This apoptosis is likely to be caused by antigen binding the immunoglobulin receptor directly. Whatever the mechanism, this phenomenon may reflect a special susceptibility of germinal centre cells to tolerance induction, which has evolved to cope with the generation of self-reactive B cells through somatic hypermutation. □

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- MacLennan, I. C. M. *A. Rev. Immun.* **12**, 117–139 (1994).
- MacLennan, I. C. M., Liu, Y. J., Oldfield, S., Zhang, J. & Lane, P. J. *L. Curr. Top. Microbiol. Immun.* **159**, 138–148 (1990).
- Jacob, J., Kassir, R. & Kelsoe, G. *J. exp. Med.* **172**, 1165–1175 (1991).
- Berek, C., Berger, A. & Apel, M. *Cell* **67**, 1121–1129 (1991).
- Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. *Nature* **354**, 389–392 (1991).
- Lalor, P. A., Nossal, G. J. V., Sanderson, R. D. & McHeyzer-Williams, M. G. *Eur. J. Immun.* **22**, 3001–3011 (1992).
- McHeyzer-Williams, M. G., McLean, M. J., Lalor, P. A. & Nossal, G. J. V. *J. exp. Med.* **178**, 295–307 (1993).
- Kuppers, R., Zhao, M., Hansmann, M. L. & Rajewsky, K. *EMBO J.* **12**, 4955–4967 (1993).
- Allen, D. *et al. Immun. Rev.* **96**, 5–22 (1987).
- Allen, D., Simon, T., Sablitzky, F., Rajewsky, K. & Cumano, A. *EMBO J.* **7**, 1995–2001 (1988).
- Berek, C. & Milstein, C. *Immun. Rev.* **96**, 23–41 (1987).
- Shlomchik, M. J., Marshak-Rothstein, A., Wolfowicz, C. B., Rothstein, T. L. & Weigert, M. G. *Nature* **328**, 805–811 (1987).
- Shlomchik, M. *et al. J. exp. Med.* **171**, 265–297 (1990).
- Diamond, B. *et al. A. Rev. Immun.* **10**, 737–757 (1992).
- Linton, P. J., Rudie, A. & Klinman, N. R. *J. Immun.* **146**, 4099–4104 (1991).
- Nossal, G. J. V., Karvelas, M. & Pulevrand, B. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3088–3092 (1993).
- Jack, R. S., Imanishi-Kari & Rajewsky, K. *Eur. J. Immun.* **7**, 559–565 (1977).
- Reth, M. G., Hammerling, G. J. & Rajewsky, K. *Eur. J. Immun.* **8**, 393–400 (1978).
- Herzenberg, L. A., Black, S. J., Tokuhisa, T. & Herzenberg, L. A. *J. exp. Med.* **151**, 1071–1087 (1980).
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. *J. Cell. Biol.* **119**, 493–501 (1992).
- Theofilopoulos, A. N. & Dixon, F. J. *Adv. Immun.* **28**, 89–220 (1978).
- Vaux, D. L. *Proc. natn. Acad. Sci. U.S.A.* **90**, 786–789 (1993).
- Nakayama, K. *et al. Science* **261**, 1548–1588 (1993).
- Veis, D. J., Sorenson, C. M., Shutter, J. R. & Korsmeyer, S. J. *Cell* **75**, 229–240 (1993).
- Hartley, S. B. *et al. Cell* **72**, 325–335 (1993).
- Nisitani, S., Tsubata, T., Murakami, M., Okamoto, M. & Honjo, T. *J. exp. Med.* **178**, 1247–1254 (1993).
- Hockenbery, D. M., Zutter, M., Hickey, W., Nahm, M. & Korsmeyer, S. J. *Proc. natn. Acad. Sci. U.S.A.* **88**, 6961–6965 (1991).
- Liu, Y. J. *et al. Eur. J. Immun.* **21**, 1905–1910 (1991).
- Smith, K. G. C., Weiss, U., Rajewsky, K., Nossal, G. J. V. & Tarlinton, D. M. *Immunity* **1**, 803–813 (1994).
- Strasser, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 8661–8665 (1991).
- Shokat, K. M. & Goodnow, C. C. *Nature* **375**, 334–338 (1995).

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Antigen-induced B-cell death and elimination during germinal-centre immune responses

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DURING an immune response, hypermutation of immunoglobulin genes in B cells proliferating within germinal centres (GCs) generates variant antibodies that react with higher affinity against either foreign or self antigens^{1–9}. Several experiments suggest that self-reactive B cells may be censored at this stage of the immune

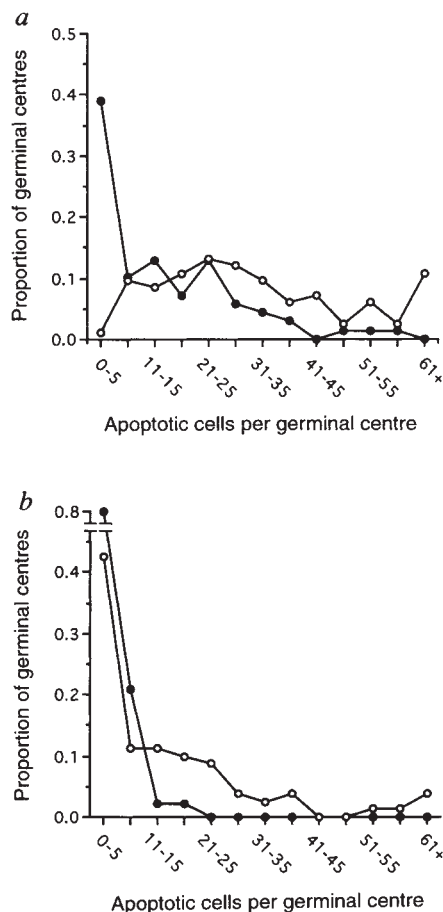
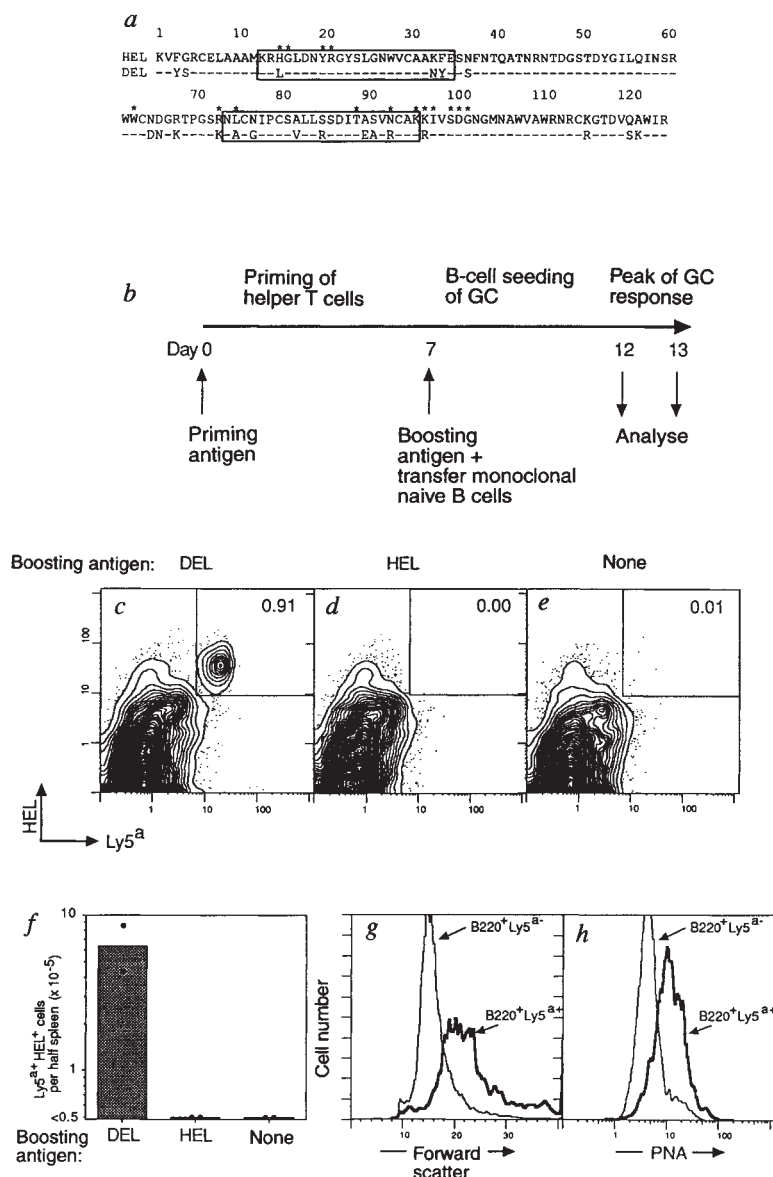


FIG. 4 The effect of *bcl-2* on apoptosis caused by soluble antigen. Mice expressing *bcl-2* constitutively in GC B cells, and their littermate controls, were immunized with NP₁₆-HSA, and injected with soluble NP₁-HSA 6 h before being killed at day 14. *a*, The littermate controls showed greatly enhanced apoptosis within a large fraction of their germinal centres. *b*, In *bcl-2* mice, there was an increase in apoptosis in a smaller, but significant fraction of the germinal centres. The residual germinal centres in the *bcl-2* mice that showed no increase in apoptosis, may represent 'background', non-antigen specific germinal centres in these mice (which were housed in conventional mouse facilities for over 24 weeks).

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FIG. 1 Seeding of germinal centre (GC) reactions with uniform lysozyme-specific B cells. **a**, Sequence comparison of HEL and DEL antigens. Dashes indicate identical amino acids, and boxed segments denote regions that are dominant T-cell epitopes in the I-A^b background¹⁴. Asterisks denote amino acids in HEL that contact HyHEL10 immunoglobulin, determined by X-ray crystal structure²⁶. The differences between HEL and DEL were exploited by priming immune responses with DEL, which had three advantages: (1) in contrast to HEL, DEL elicits a strong helper-T-cell response in B6 mice, primarily due to a F→Y mutation at position 3 which affects antigen processing¹⁴; (2) DEL differs considerably from HEL within the dominant T-cell epitopes defined in HEL¹⁴, providing a pair of antigens that would be expected to crossreact poorly at the helper-T-cell level; and (3) DEL binds to the immunoglobulin expressed by the transgenic B cells with 1,000-fold lower affinity than HEL¹⁵, thus better approximating the binding affinity of B cells during primary antibody responses. **b**, Experimental protocol used to generate a synchronous burst of lysozyme-specific GC B cells. **c–e**, FACS analysis of spleen cells from DEL-primed B6 mice on day 12 of the immune response, 5 days after transfer of Ly5^{a+} immunoglobulin-gene transgenic cells and boosting with DEL (**c**), HEL (**d**), or no additional antigen (**e**). Spleen cells were analysed by three-colour flow cytometry for cell-surface lysozyme-binding receptors (HEL), and the B220 and Ly5^a surface markers. The profiles shown were first gated on B220⁺ cells. The percentage of ungated nucleated cells falling within the B220⁺ HEL⁺ Ly5^{a+} window is shown; contours are 1% probability intervals. **f**, Total number of Ly5^{a+} B220⁺ HEL⁺ cells in the spleen of pairs of mice treated and analysed as shown in (**c–e**). **g–h**, Cell size (forward scatter, **g**), and staining with peanut agglutinin (PNA, **h**) of Ly5^{a+} B220⁺ transgenic cells in DEL-primed and boosted recipients. Overlaid histograms show forward scatter or PNA staining for transferred cells (Ly5^{a+} B220⁺, thick lines) compared to endogenous non-transgenic B cells (Ly5^{a+} B220⁺, thin lines).

METHODS. Peking duck eggs were obtained from Reichardt's Duck Farm (Petaluma, California). DEL was purified as described²⁷. A mixture of DEL A and B (ref. 27) was used for immunization and boosting. Immunoglobulin-gene transgenic cells were F₁ offspring from B6 strain MD4 transgenic mice crossed with the congenic B6-Ly5^a strain. Recipients were 8- to 14-week-old B6 mice primed with 100 µg DEL in Ribi adjuvant (MPL+TDM+CWS Emulsion, Ribi ImmunoChem Research, Hamilton, MT) given intraperitoneally. After 7 days, 1 × 10⁷ sex-matched immunoglobulin-gene transgenic spleen cells in 0.3 ml RPMI-1640 medium with 2% FCS were injected intravenously in combination with 100 µg DEL or HEL, or in medium with no added antigen. Mice were killed after 5 days; half the spleen was used for immunohistochemistry, and the other half for three-colour flow cytometry as described^{23,28}. Staining with peanut agglutinin used PNA-FITC (E-Y Laboratories, San Mateo, California). Staining for B220, Ly5^a and HEL-binding B cells was performed as previously described²⁸.

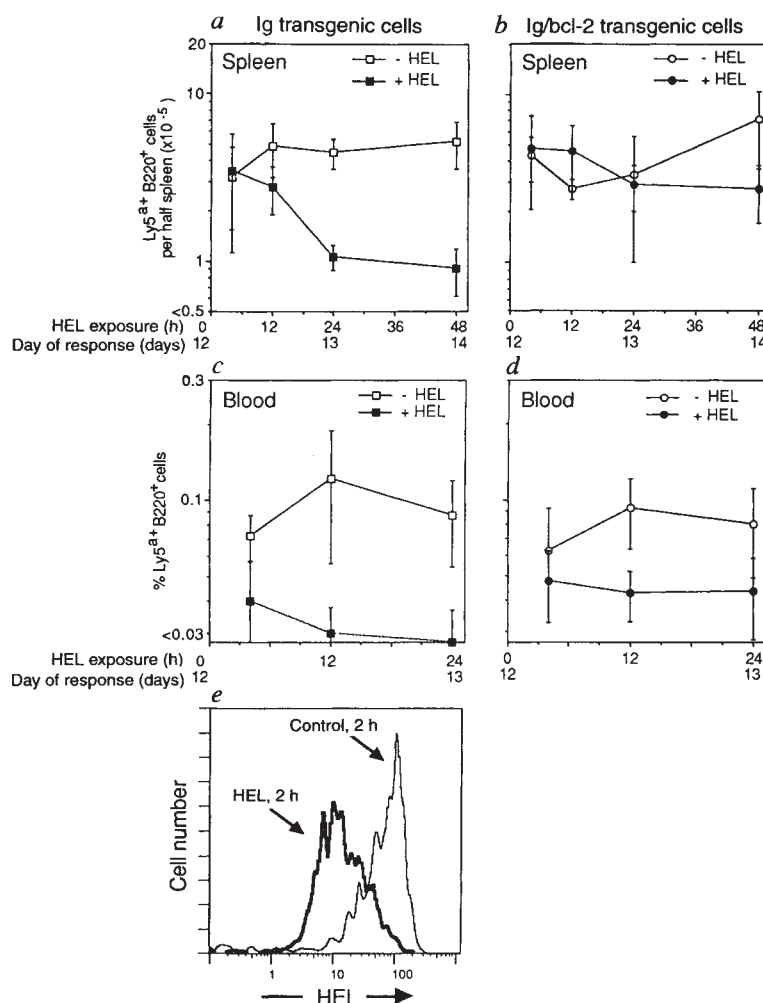


response^{10–12}, but the rarity of these cells and the dynamic nature of GC reactions have prevented direct analysis. We have developed a new approach to visualize the fate of antigen-specific B cells during GC reactions by seeding an ongoing immune response with lysozyme-specific B cells from immunoglobulin-gene transgenic animals. Administration of soluble antigen at the peak of the GC response rapidly eliminates lysozyme-specific GC B cells in two waves of apoptosis, one within the GC and a second in cells that have redistributed to lymphoid zones that are rich in T cells. Elimination of these cells is inhibited by constitutive expression of the follicular lymphoma proto-oncogene *bcl-2*. These findings reveal censoring steps that may normally prevent affinity maturation of autoantibodies to systemic autoantigens, and might be used by pathogenic microorganisms or in clinical strategies to interfere with antibody responses.

To track GC cell selection, a burst of proliferating GC B cells with a uniform immunoglobulin specificity was produced

by seeding a primary antibody response with lysozyme-specific B cells from an immunoglobulin-gene transgenic mouse¹³. An immune response was initiated by priming C57BL/6 mice with the antigen duck egg lysozyme (DEL), which was chosen to exploit sequence differences from hen egg lysozyme (HEL)^{14,15} (Fig. 1a). After 7 days, when T-cell help would be well developed and GCs beginning to form¹⁶, 10⁷ immunoglobulin-gene transgenic spleen cells were introduced together with a boosting dose of DEL by intravenous injection (Fig. 1b). The transferred B cells were tracked by their lysozyme-binding receptors and a unique Ly5^a surface marker. They multiplied rapidly (Fig. 1c,f), and peak numbers occurred on days 12 to 13 of the response when the seeded cells were large (Fig. 1g), stained brightly with peanut agglutinin (Fig. 1h) and dominated the GC reactions. Transferred B cells did not proliferate detectably in DEL-primed mice that were boosted with HEL despite the 1,000-fold higher affinity of the B cells for HEL antigen (Fig.

FIG. 2 Elimination of seeded lysozyme-specific GC B cells after binding HEL antigen. Groups of recipient mice were primed with DEL antigen, seeded with transgenic B cells and boosted with DEL antigen as described in Fig. 1, except that immunoglobulin-gene transgenic cells (a, c, e) or Ig/bcl-2 transgenic cells (b, d) were used. Five days after B-cell seeding (day 12 of the immune response), the mice received an intravenous injection of 5 mg HEL antigen (+HEL) or medium alone (-HEL). At the indicated times after HEL treatment, groups of 3 to 5 mice in each treatment group were killed and B220⁺ Ly5^{a+} cells enumerated in spleen (a, b) and blood (c, d) by flow cytometry. Means and standard deviations for each group of mice are shown. The data shown are pooled from two separate experiments involving the same four treatment groups. Equivalent data were obtained in six other experiments with immunoglobulin-gene transgenic cells and three experiments comparing Ig/bcl-2 cells. e, Histograms showing HEL staining of Ly5^{a+} B220⁺ cells 2 h after injection of HEL (thick line) or medium alone (thin line). Staining is by the sandwich technique which shows the total number of cell surface receptors regardless of the fraction occupied *in vivo*. **METHODS.** B6 recipients were primed and boosted as described in Fig. 1. They were seeded with 10⁷ spleen cells from sex matched (B6 × B6-Ly5^a)F₁ mice that were either MD-4 immunoglobulin-gene transgenic mice or also carried the Eμ-bcl-2-22 transgene¹⁷. On day 12 of the response, mice received 300 μl RPMI-1640 medium either alone or containing 5 mg HEL, via the lateral tail vein. Mice were killed with CO₂ at the indicated times, heart blood was collected in Alsever's solution and erythrocytes removed by NH₄Cl lysis, and blood leukocytes and half of the spleen was used for flow cytometry. The other half of the spleen was divided for frozen section and paraffin embedding.



1d, f), possibly because HEL-derived peptides were not recognized by DEL-specific helper T cells.

To model the fate of GC B cells that become strongly reactive to circulating autoantigens as a result of immunoglobulin gene hypermutation, soluble HEL antigen was injected on day 12 of the response to expose the GC B-cell population to soluble HEL antigen in an abrupt and synchronous manner. Cell-surface antigen receptors were rapidly modulated on splenic B220⁺ Ly5^a B-cell blasts following HEL antigen (Fig. 2e), but the seeded cells were not markedly reduced in number until after 24 hours (Fig. 2a). After 48 hours the number remaining was at the lower limit of detection by flow cytometry. Lysozyme-reactive B-cell blasts in the bloodstream of HEL-treated mice disappeared well before any reduction in the spleen (Fig. 2c), implying that elimination from the spleen might reflect cell death *in situ* rather than emigration via the blood. This was confirmed by seeding parallel groups of mice with immunoglobulin-gene transgenic B cells that also carried a constitutively expressed bcl-2 transgene which inhibits cell death¹⁷. The number of splenic lysozyme-reactive B-cell blasts was not reduced 24 hours after HEL injection in mice that had received Ig/bcl-2 transgenic cells, and only a twofold reduction occurred after 48 hours (Fig. 2b).

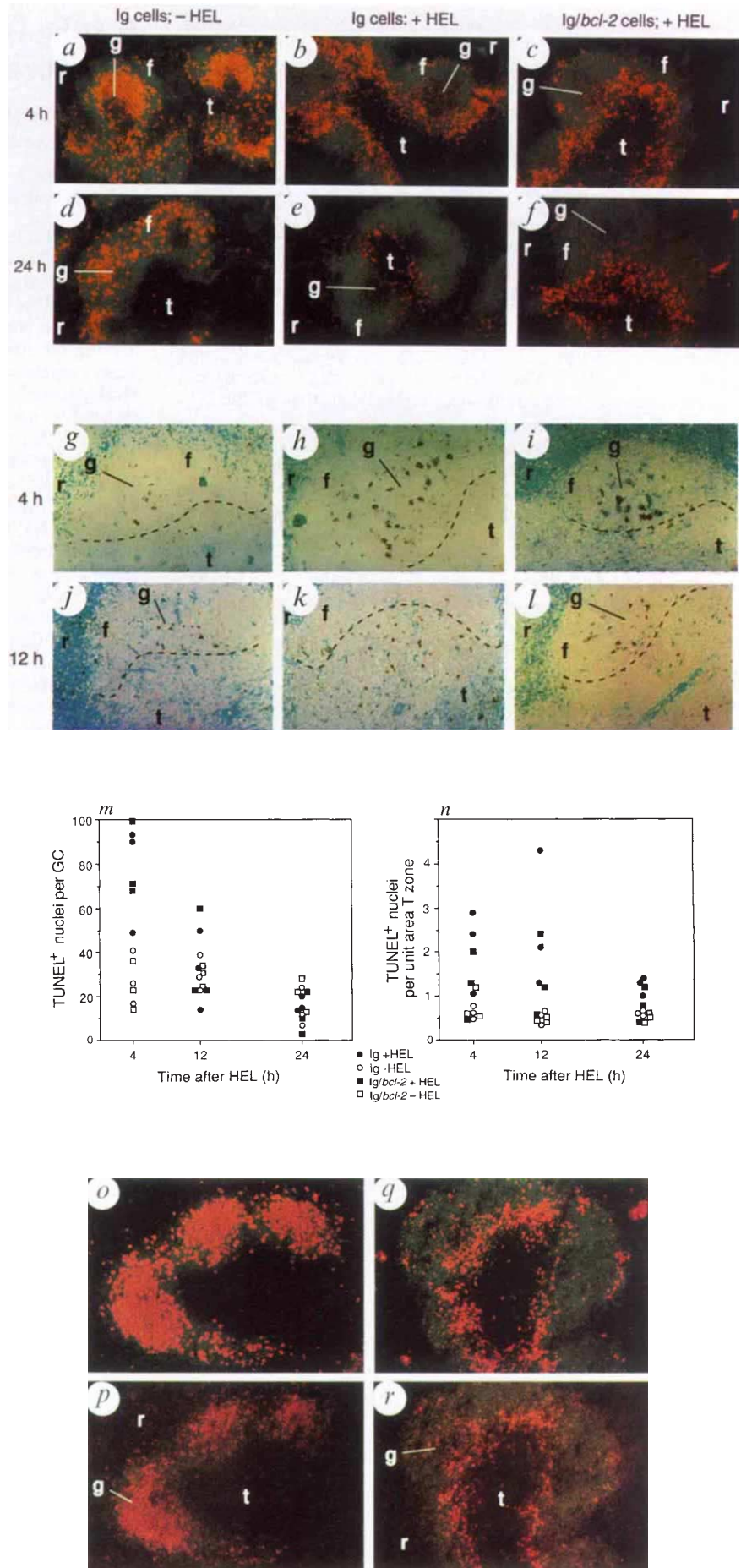
The precise sites of B-cell death were explored by locating either lysozyme-binding Ly5^{a+} B cells in spleen cryosections by immunofluorescent staining, or dying cells in paraffin sections by the TUNEL method¹⁸ to detect DNA fragmentation. In the absence of HEL, lysozyme-binding B cells were located predominantly in the apical zones of GCs with scattered cells in the surrounding mantle and marginal zones (Fig. 3a, d), and occasional TUNEL⁺ nuclei were clustered in tingible body macro-

phages in GCs (Fig. 3g, j). Within 4 hours of HEL injection, however, few lysozyme-binding cells remained in the GCs (Fig. 3b) and TUNEL⁺ nuclei were markedly increased in this site, many being clustered in groups of 10 to 20 nuclei within prominent tingible-body macrophages (Fig. 3h, m). Large numbers of lysozyme-binding B cells were also redistributed to the outer part of the T-cell zones at this time (Fig. 3b) and after 12 hours (data not shown), but few remained by 24 hours after HEL treatment (Fig. 3e). Staining of serial sections for Ly5^a showed comparable cell distributions (Fig. 3o-r), confirming that the transferred B cells were rapidly depleted from the GCs and concentrated in the T-cell zones following soluble HEL administration. TUNEL⁺ nuclei were more frequent in the T-cell zone 4 to 12 h after HEL treatment (Fig. 3k, n), but had mostly disappeared by 24 hours consistent with the elimination of HEL-

FIG. 3 Location of B-cell death. Spleen sections from the mice analysed in Fig. 2 were analysed by immunofluorescence to detect seeded B cells (a-f, o-r) and by the TUNEL method to detect apoptotic cells with fragmented DNA (g-n). a-f, Two-colour fluorescence micrographs of 8-μm spleen cryosections stained for HEL-binding cells (orange) and a pan-B cell marker, CD79β (B29/Igβ; green). T-cell zones (T), germinal centres (G), follicular mantle zones (F) and red pulp (R) are indicated. In recipients of immunoglobulin-gene transgenic cells that did not receive HEL antigen (-HEL), HEL-binding cells are found predominantly in the apical zones of GCs with scattered cells in mantle and marginal zones (a, d). Cells in the basal zone of GCs carry few immunoglobulin receptors of any type^{16,20}, as they also lack staining for CD79β (green). Note that in a the T-cell zone is sectioned tangentially and the scattered

HEL-binding cells near the T-cell zone are in follicular mantle zones. In mice treated with HEL (+HEL), few HEL-binding cells remain in the GC at 4 h (b, c), and none are detected after 24 h (e, f), and none are detected after 24 h (e, f), and most HEL-binding cells are concentrated in the outer part of the T-cell zone. g–l, Transmission micrographs of TUNEL staining in 3- μ m paraffin sections showing apoptotic nuclei containing nicked DNA (brown), counterstained with methyl green. The edge of the T-cell zone is outlined with broken lines, and was determined by viewing with phase contrast to detect the characteristic interdigitating dendritic cells of the T-cell zone and from adjacent serial sections stained with haematoxylin and eosin. In the absence of HEL (g, j), few apoptotic nuclei are present in the GCs and very few are in the T-cell zones. By contrast, large numbers of clustered apoptotic nuclei are present in GCs 4 h after HEL treatment (h, i). Apoptotic nuclei are also scattered through the T-cell zones 12 h after HEL treatment (k, l). Equivalent results to those shown in a–l were obtained from each of the mice shown in Fig. 2, and in four separate experiments. m, n, Mean number of apoptotic nuclei in GCs (m) and T-cell zones (n) of individual mice at each time point. o–r, Two-colour immunofluorescent staining as in a–g, except that orange staining of serial sections represents either HEL-binding (o, q) or staining for Ly5^a (p, r). Note that staining for Ly5^a is not as bright as HEL staining and hence is less sensitive in tissue sections.

METHODS. One-quarter of each spleen from the mice shown in Fig. 2 was cut into 5-mm sections and snap-frozen in OCT (Biomed, Foster City, California) for cryosections. Another quarter was cut into 5-mm sections, fixed in 10% neutral buffered formalin, and embedded in paraffin. Immunofluorescence staining was as described²⁹. HEL staining used a three-layer sandwich of sequential staining with HEL, HyHEL9-biotin and streptavidin-PE. Ly5^a staining was with biotinylated AS-20 followed by streptavidin-PE. CD79 β was detected with affinity-purified rabbit antiserum raised against a glutathione S-transferase (GST)–B29 cytoplasmic tail fusion protein (generous gift of J. G. Cyster) followed by donkey anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; Jackson ImmunoResearch). Two-colour fluorescence was visualized and photographed through a dual filter system: excitation 480–500 nm/emission 517–532 nm (FITC) and excitation 545–578 nm/emission 585–620 nm (phycoerythrin, PE; Chroma, Brattleborough, VT). The method of Gavrieli *et al.* was used for TUNEL staining with minor modifications¹⁸. Paraffin was removed using Hemo-De (Fisher Scientific) under the same conditions as described for use of xylene. Endogenous peroxidase activity was blocked with methanol: 3% aqueous H₂O₂ for 10 min. Following the TUNEL reaction, the sections were counterstained with an aqueous solution of 0.5% lissamine green for 45 s, followed by 3 rinses in water, 3 rinses in *n*-butanol, and dehydration using Hemo-De. For quantitation, slides were coded, randomized, white pulp cords sketched under phase contrast and by viewing haematoxylin and eosin-stained serial sections, and apoptotic nuclei counted in 10–20 GCs and 4–6 T-cell zones from each mouse. The area of each counted T-cell zone was measured using a grid in one eyepiece containing 56 μ m $\times$ 56 μ m squares, and the number of apoptotic nuclei in each T-cell zone was divided by the number of 56 μ m $\times$ 56 μ m squares to give a value per unit area.



binding Ly5^{5+} cells by this time. Equivalent changes occurred after HEL treatment in recipients of Ig/*bcl-2* transgenic cells (Fig. 3c, f, i, l, m), except that many lysozyme-binding Ly5^{5+} cells remained in the T-cell zone after 24 hours (Fig. 3f) and fewer TUNEL⁺ nuclei were present in this site (Fig. 3l, n).

These results show that circulating antigen causes apoptosis and elimination of specific GC B cells in two waves. The data do not address how apoptosis is triggered in each case, but it is tempting to speculate that the rapid cell death, within 4 hours in GCs, may reflect early centrocytes emerging from the GC basal dark zones that were prevented from binding DEL antigen displayed on follicular dendritic cells (FDC), because recognition of FDC-bound antigen may deliver short-term survival signals to centrocytes^{19, 21}. The *bcl-2* transgene was unable to block this phase of HEL-induced B-cell death (Fig. 3i, m), but may inhibit comparable death of GC B cells in response to the hapten nitrophenyl (NP)²². The affinity for HEL in the GC B cells studied here is 100 to 1,000-fold higher than that of the NP-reactive cells^{3, 5}, and may thus exceed the capacity of *bcl-2* to interfere with apoptosis at this selection step. The slower phase of B-cell disappearance in the T-cell zones, which was inhibited by *bcl-2*, may represent older centrocytes that had recognized FDC-bound DEL antigen and received survival signals before HEL exposure. Their apparent migration to the T-cell zones resembles the redistribution of marginal-zone memory B cells following restimulation with antigen¹⁶, and implies that antigen-receptor engagement induces a tropism of B cells towards T-cell zones. Premature death of primed HEL-binding cells in the T-cell zone resembles competitive elimination of self-reactive B cells in the preimmune repertoire²³, and may reflect exclusion from follicular niches and absence of T-cell help²³. The two censoring steps identified here may normally eliminate B cells that, through hypermutation, bind systemic autoantigens such as DNA too strongly^{6, 8, 9, 24}. The ability to abort B-cell affinity maturation with soluble antigen also suggests clinical strategies to curtail undesirable antibody responses, and might be exploited by pathogenic organisms, such as malaria, which produce appreciable amounts of circulating antigen²⁵. □

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- Weigert, M. G., Cesari, I. M., Yonkovich, S. J. & Cohn, M. *Nature* **228**, 1045–1047 (1970).
- Tonegawa, S. *Nature* **302**, 575–581 (1983).
- Kocks, C. & Rajewsky, K. *A. Rev. Immun.* **7**, 537–559 (1989).
- Jacob, J., Kelsøe, G., Rajewsky, K. & Weiss, U. *Nature* **354**, 389–392 (1991).
- Berek, C., Berger, A. & Apel, M. *Cell* **67**, 1121–1129 (1991).
- Diamond, B. & Scharff, M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5841–5844 (1984).
- Shlomchik, M. J., Marshak-Rothstein, A., Wolfowicz, C. B., Rothstein, T. L. & Weigert, M. G. *Nature* **328**, 805–811 (1987).
- Shlomchik, M. et al. *J. exp. Med.* **171**, 265–292 (1990).
- Diamond, B. et al. *A. Rev. Immun.* **10**, 731–757 (1992).
- Linton, P.-J., Rudie, A. & Klinman, N. R. *J. Immun.* **146**, 4099–4104 (1991).
- Dintzis, H. M. & Dintzis, R. Z. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1113–1117 (1992).
- Nossal, G. J. V., Karvelas, M. & Pulendran, B. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3088–3092 (1993).
- Goodnow, C. C. et al. *Nature* **334**, 676–682 (1988).
- Gammon, G. et al. *Immunol. Rev.* **98**, 53–73 (1987).
- Lavoie, T. B., Drohan, W. N. & Smith-Gill, S. J. *J. Immun.* **148**, 503–513 (1992).
- Liu, Y.-J., Zhang, J., Lane, P. J. L., Chan, E. Y.-T. & MacLennan, I. C. M. *Eur. J. Immun.* **21**, 2951–2962 (1991).
- Strasser, A. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8661–8665 (1991).
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. *J. Cell Biol.* **119**, 493–501 (1992).
- Liu, Y.-J. et al. *Nature* **342**, 929–931 (1989).
- MacLennan, I. C. M. *A. Rev. Immun.* **12**, 117–139 (1994).
- Gray, D., Dullforce, P. & Jainandasing, S. J. *exp. Med.* **180**, 141–155 (1994).
- Pulendran, B., Kannanarokis, G., Nouri, S., Smith, K. G. C. & Nossal, G. J. V. *Nature* **375**, 331–334 (1995).
- Cyster, J., Hartley, S. & Goodnow, C. C. *Nature* **371**, 389–395 (1994).
- Radice, M. Z. & Weigert, M. A. *Rev. Immun.* **12**, 487–520 (1994).
- Kemp, D. J., Coppel, R. L. & Anders, R. F. *A. Rev. Microbiol.* **41**, 181–208 (1987).
- Padian, E. A. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5938–5942 (1989).
- Prager, E. M. & Wilson, A. C. *J. biol. Chem.* **246**, 523–530 (1971).
- Cooke, M. P. et al. *J. exp. Med.* **179**, 425–438 (1994).
- Lagasse, E. & Weissman, I. L. *Blood* **79**, 1907–1915 (1992).

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A role for Rac in Tiam1-induced membrane ruffling and invasion

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RHO-LIKE GTPases have been implicated in the regulation of the actin cytoskeleton which controls the morphology, adhesion and motility of cells^{1–3}. Like Ras proteins, they become activated when bound GDP is exchanged for GTP, a process catalysed by GDP-dissociation stimulator (GDS) proteins⁴. Several GDS proteins specific for Rho-like GTPases have been identified^{5–8}. Most of these contain a conserved catalytic domain, the DBL-homology (DH) domain⁹, and activate Cdc42 or Rho but not Rac^{5–8}. We have isolated the invasion-inducing *Tiam1* gene, which also encodes a protein with a DH domain¹⁰. Here we show that *Tiam1* is a GDS protein for Rho-like GTPases *in vitro*. In fibroblasts, *Tiam1* induces a similar phenotype as constitutively activated (V12)Rac1, including membrane ruffling, and this is inhibited by dominant negative (N17)Rac1. Moreover, T-lymphoma cells expressing V12Rac1 become invasive, indicating that the *Tiam1*–Rac signalling pathway could be operating in the invasion and metastasis of tumour cells.

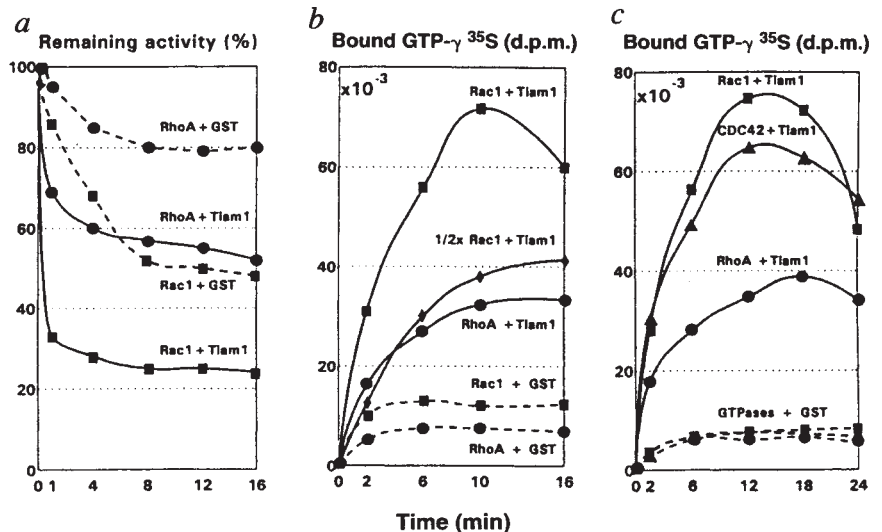
To investigate whether *Tiam1* is a GDS for Rho-like GTPases, *Tiam1*–glutathione *S*-transferase (GST) and Myc-tagged RhoA and Rac1 fusion proteins were purified from transfected COS-7 cells. Isolated Rac1 and RhoA proteins were preloaded with ³H-GDP and incubated with *Tiam1*–GST or GST alone to determine the release of bound ³H-GDP as described^{6, 11}. Incubation of Rac1 with *Tiam1*–GST resulted in a pronounced release of ³H-GDP when compared to the incubation of Rac1 with GST (Fig. 1a). A similar, though slower, release was observed for RhoA (Fig. 1a). The *Tiam1*-induced release of ³H-GDP was evident within the first 5 minutes of incubation. To substantiate these findings further, we analysed the effect of *Tiam1* on the exchange of preloaded GDP for GTP- γ ³⁵S on Rac1 and RhoA. Indeed, *Tiam1* acted as a more potent GDS for Rac1 than for RhoA as both the rate of exchange and the total amount of bound GTP- γ ³⁵S were higher for Rac1 than for RhoA (Fig. 1b). Similar results were obtained with GST–RhoA and GST–Rac1 fusion proteins isolated from *Escherichia coli* (Fig. 1c). In the latter experiment we included GST–hCdc42, a Rac-related GTPase¹², which was also efficiently activated by *Tiam1*. These experiments indicate that *Tiam1* is a GDS protein for Rho-like GTPases and that *in vitro* *Tiam1* activates Rac1 and hCdc42 and, to a lesser extent, RhoA.

We next determined whether *Tiam1* could also activate Rac and Rho proteins in fibroblasts. Constitutively activated RhoA (V14RhoA) induces focal contacts and stress fibre formation in 3T3 cells¹³, whereas V12Rac1 induces the formation of actin filaments at the plasma membrane leading to membrane ruffling¹⁴. *Tiam1*-encoding constructs were transfected into NIH3T3 cells and the resulting transfectants were compared with V12Rac1- and V14RhoA-transfected cells. Cells that transiently expressed the full-length or N-terminally truncated *Tiam1* (Δ N-*Tiam1*) showed extensive membrane ruffling (Fig. 2a, b), as was found for V12Rac1-transfected (Fig. 2d) or -microinjected cells¹⁴. Membrane ruffling was observed in more than 80% of the *Tiam1*-transfected cells and hardly at all (less than 5%) in untransfected cells. V14RhoA induced the formation of large bundles of stress fibres (Fig. 2c), which was not seen in *Tiam1*-transfected cells. The membrane ruffling in established Δ N-*Tiam1*-transformed NIH3T3 cell lines, was inhibited in most (>70%) of the cells that co-expressed dominant negative Rac1

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FIG. 1 Tiam1 is a GDS protein for Rho-like GTPases *in vitro*. **a**, Dissociation of ^3H -GDP from Myc-RhoA (●) and Myc-Rac (■) was measured at the indicated times in the presence of Tiam1-GST (solid line) or GST (dotted line). **b** and **c**, Kinetics of the association of GTP- $\gamma^{35}\text{S}$ to GDP-loaded Myc-RhoA (●) and Myc-Rac1 (■ and ◆) (**b**), and to GDP-loaded GST-RhoA (●), GST-Rac1 (■) and GST-Cdc42 (▲) (**c**), in the presence of Tiam1-GST (solid line) or GST (dotted line). Note the remarkable instability of nucleotide-bound Rac1, as previously reported¹¹.

METHODS. Myc-tagged fusion proteins were isolated from transfected COS-7 cells on a 9E10 antibody²²-coupled Sepharose column. Tiam1-GST and GST were purified from COS-7 cells as described²³. *E. coli*-derived GST-tagged GTPases were a gift from T. Miki. N-terminally truncated Tiam1 ($\Delta\text{N-Tiam1}$) was used in all assays as the full-length protein could not be isolated (see also Fig. 2). Purified Myc-tagged GTPases (20 pmol) were preloaded with [^3H]GDP (30 μM ; 12.7 Ci mmol⁻¹) in 10 μl as described¹¹. Four μl of the preloaded GTPase was added to 16 μl of exchange mixture, which contained 2 pmol Tiam1-GST or GST, 1 mM GTP and 60 $\mu\text{g ml}^{-1}$ BSA in exchange buffer¹¹. At the indicated times, samples of 3 μl were analysed^{6,11}. Incubation with EDTA released about 90% of the prebound [^3H]GDP. For the exchange reactions towards GTP- $\gamma^{35}\text{S}$, GST-tagged GTPases (20 pmol) were preloaded with 0.5 μM GDP. Myc-tagged GTPases (20 pmol (● and ■) or 10 pmol (◆)) were preloaded with 0.5 μM GDP in exchange buffer containing 200 $\mu\text{g ml}^{-1}$



BSA. Tiam1-GST and GST were preincubated for 10 min with 0.25 μM GTP- $\gamma^{35}\text{S}$ (1,393 Ci mmol⁻¹) and 2.25 μM GTP- γS , followed by addition of the GDP-loaded GTPases. The amount of GTP- $\gamma^{35}\text{S}$ bound to Tiam1-GST (5–7,000 d.p.m.) and GST (1,000 d.p.m.), in the absence of GTPases, was subtracted from the original values. Representative results of at least three independent experiments are shown.

(N17Rac1) (Fig. 2e, f), indicating that Rac1 acts downstream of Tiam1. Treatment with C3 ADP-ribosyl transferase, which inactivates Rho¹⁵, did not affect membrane ruffling but caused dissociation of most basal stress fibres in control and Tiam1-transfected cells (data not shown). This indicates that Tiam1-induced membrane ruffling is independent of RhoA activity¹⁶. Like Rac1, both full-length and truncated Tiam1 protein were

present in the cytoplasm and co-localized with F-actin in membrane ruffles (Fig. 2). In phase-contrast microscopy, Tiam1-transfected cells showed a typical sickle- or pancake-shaped morphology. They were often multinucleated, contained many pinocytotic vesicles and showed extensive membrane ruffling (Fig. 3), a phenotype similar to that of V12Rac1-injected cells¹⁴. From these experiments we conclude that Tiam1 activates Rac but not Rho in fibroblasts.

Transfection of Tiam1 cDNA into non-invasive T-lymphoma cells induced invasiveness as assessed on monolayers of fibroblasts¹⁰. If Tiam1 activates Rac *in vivo*, the introduction of constitutively activated V12Rac1 into non-invasive T-lymphoma cells might also induce invasiveness. To test this hypothesis, we

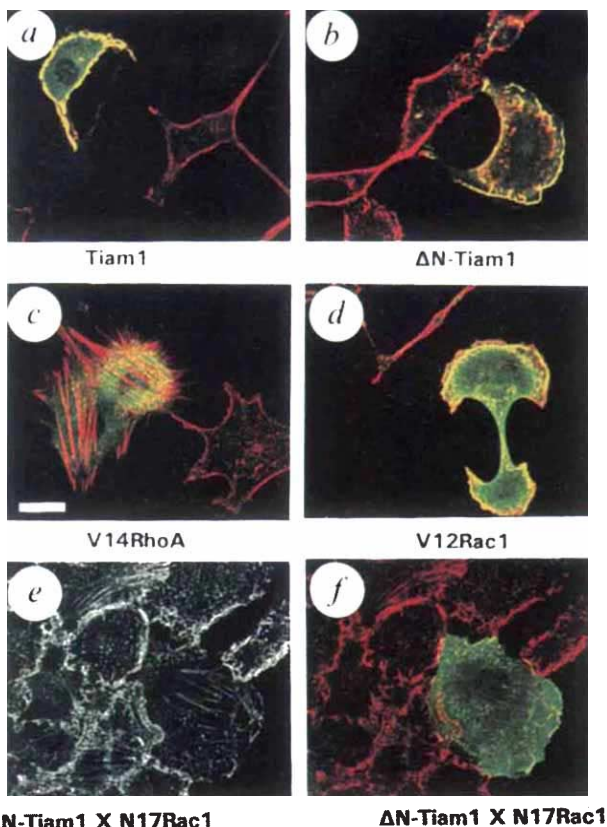
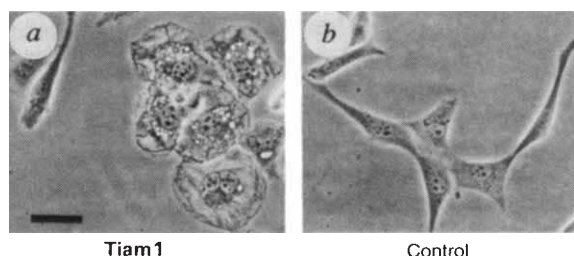


FIG. 2 Tiam1 and V12Rac1 induce membrane ruffles in NIH3T3 cells. Confocal microscopic images of NIH3T3 cells transiently expressing full-length Tiam1 (**a**), N-terminally truncated Tiam1 ($\Delta\text{N-Tiam1}$) (**b**), V14RhoA (**c**), V12Rac1 (**d**), or an established $\Delta\text{N-Tiam1}$ -expressing cell line that was transfected with N17Rac1 (**e**, **f**). Expressed proteins (only N17Rac1 and not Tiam1 in **f**) were visualized with fluorescein isothiocyanate (FITC; green), and F-actin with tetramethylrhodamine-5 (and -6) isothiocyanate (TRITC) phalloidin (red). The green and red fluorescence images were recorded separately and merged. Co-localization is in yellow. In **e**, only the phalloidin staining of F-actin is shown.

METHODS. NIH3T3 cells were transfected using the calcium phosphate precipitation technique for 6 h²⁴ with 4 μg of pEXV expression constructs encoding Myc-tagged V14RhoA or V12Rac1 (from A. Hall), or 4 μg of pRc/RSV-neo (Invitrogen) encoding full-length Tiam1 or N-terminally truncated Tiam1 (PDH¹⁰). The latter construct encodes an identical but untagged protein to that used for *in vitro* analysis (Fig. 1). An established cell line expressing N-terminally truncated Tiam1 was transfected with 4 μg Myc-tagged N17Rac1 in pEXV. After 12 h or 24 h (**e**, **f**) in fresh medium cells were fixed in formaldehyde (3%) and stained with either the anti-Myc-tag monoclonal antibody 9E10 (ref. 22) or a Tiam1-specific polyclonal rabbit antiserum to identify V14RhoA-, V12Rac1- and N17Rac1- or Tiam1-expressing cells, respectively. Antibodies were visualized with FITC-labelled secondary antibodies (Zymed) and cells were stained simultaneously for F-actin with TRITC-labelled phalloidin (Molecular Probes) at a concentration of 2 units per ml. Bar represents 15 μm .



Tiam1

Control

FIG. 3 Tiam1-induced morphological transformation of fibroblasts. Phase-contrast images of Tiam1-transfected NIH3T3 cells (a) and untransfected control cells (b). Tiam1-transfected cells are flat and often sickle- or pancake-shaped. Note the extensive membrane ruffling and formation of pinocytotic vesicles.

METHODS. A full-length Tiam1-expression construct was transfected as described in Fig. 2 legend and cells were cultured for 5 days. Bar represents 15 μ m.

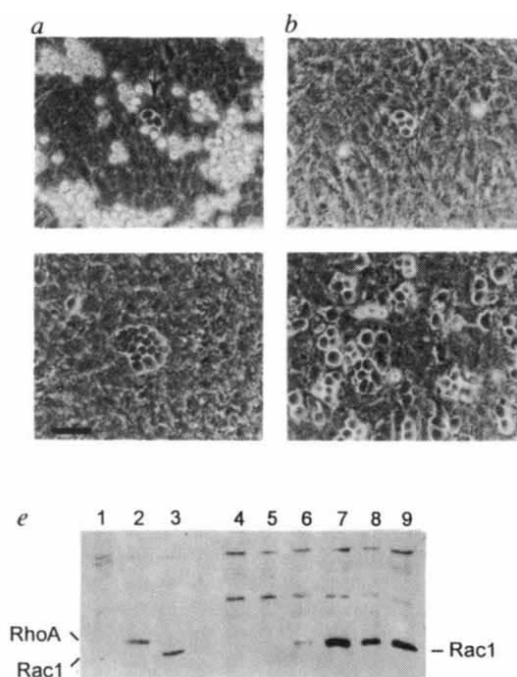


FIG. 4 Induction of invasiveness in BW5147 T-lymphoma cells by V12Rac1. a, Phase-contrast microscopic image of V12Rac1-infected lymphoma cells layered on a monolayer of rat embryonic fibroblasts. Infiltrated cells are indicated (arrow). b, As a, after washing away non-infiltrated cells. c, Infiltrated cells were allowed to proliferate for 3 d. d, Isolated infiltrated cells were expanded and tested again on a monolayer to confirm that these cells were invasive. e, Western blot analysis of lysates of NIH3T3 cells infected with LacZ (lane 1), V14RhoA (lane 2) or V12Rac1 (lane 3), and lysates of uninfected BW5147 cells (lane 4), of V12Rac1-infected BW5147 cells before layering on fibroblast monolayers (lane 5) and of four independently isolated infiltrated cell clones (lanes 6–9). The fibroblast cells were efficiently infected (25%) and the V12Rac1 and V14RhoA proteins are clearly visible. V12Rac1 expression was not detectable in the original infected BW5147 population (lane 5) as the infection efficiency of these cells was only 0.5%, but was clearly seen in expanded infiltrated cells (lanes 6–9). METHODS. Myc-tagged V12Rac1 and V14RhoA cDNAs were substituted for the LacZ gene in the pMFG-S-LacZ²⁵ retroviral expression vector (Somatix). The resulting plasmids were used to produce V12Rac1-, V14RhoA- or LacZ-transducing retroviruses in BOSC23 packaging cells as described²⁶. BW5147 T-lymphoma cells or NIH3T3 cells (3×10^5 cells) were infected by culturing them for 18 h in 2.5 ml cell-free BOSC23 supernatants²⁶. Thereafter, cells were incubated in fresh medium for another 48 h and analysed for protein expression by X-gal and antibody staining. The invasive capacity of infected BW5147 cells was determined as described¹⁰. Cells from colonies of infiltrated cells were isolated with a cloning cylinder. Lysates of 10^5 cells were analysed by western blot analysis using the anti-Myc tag monoclonal antibody 9E10 (ref. 22). Bar represents 50 μ m.

infected BW5147 lymphoma cells with retroviral vectors that contained V12Rac1, V14RhoA or LacZ cDNAs. Retroviral transduction of V12Rac1 yielded invasive cells (Fig. 4a–d), whereas no invasive cells were obtained after transduction of V14RhoA or LacZ (not shown). All viral suspensions were equally infectious as determined by antibody staining and western blot analysis (Fig. 4e). Infiltrated cells strongly expressed the exogenous V12Rac1 protein, whereas non-invasive cells did not (Fig. 4e). Thus, Tiam1-induced invasiveness of T-lymphoma cells is mimicked by expression of constitutively activated Rac1.

Rac is known to play a central role in signalling pathways that regulate actin polymerization at the plasma membrane in response to distinct extracellular stimuli¹⁴. Important regulators of Rac identified so far are Rho–GDI and several GTPase-activating proteins¹⁷. From our data we conclude that Tiam1 functions as an activator (GDS) of Rac *in vitro* as well as *in vivo*. The accumulation of Tiam1 at the plasma membrane suggests that translocation of Tiam1 is required to activate Rac, in a way analogous to the activation of Ras by Sos¹⁸. Tiam1 may be essential in Rac-mediated signalling pathways, an idea supported by the ubiquitous expression of Tiam1 (ref. 19). Whether Tiam1 is also a regulatory component of the Rac-dependent NADPH oxidase system in neutrophils²⁰ remains to be investigated. A conclusion from this study is that Rac is involved in regulating the invasive capacity of tumour cells because expression of both Tiam1 and constitutively activated V12Rac1 lead to invasive T-lymphoma cells. Tiam1 also induces the tumorigenic phenotype in NIH3T3 cells (manuscript in preparation), as does V12Rac1 (ref. 21). Aberrations in the Tiam1–Rac pathway could therefore have consequences for tumorigenesis and metastasis in general. □

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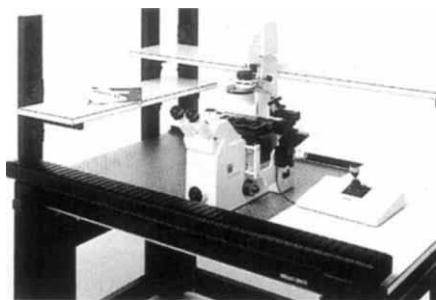
- Hall, A. *Molec. Biol. Cell* **3**, 475–479 (1992).
- Tominaga, T. *et al. J. Cell Biol.* **120**, 1529–1537 (1993).
- Takaishi, K. *et al. Molec. cell. Biol.* **13**, 72–79 (1993).
- Boguski, M. S. & McCormick, F. *Nature* **366**, 643–654 (1993).
- Hiraoka, K. *et al. Biochem. biophys. Res. Commun.* **182**, 921–930 (1992).
- Hart, M. J. *et al. Nature* **354**, 311–314 (1991).
- Zheng, Y., Cerione, R. & Bender, A. *J. Biol. Chem.* **269**, 2369–2372 (1994).
- Hori, Y. *et al. EMBO J.* **13**, 4776–4786 (1994).
- Hart, M. J. *et al. J. Biol. Chem.* **269**, 62–65 (1994).
- Habets, G. G. M. *et al. Cell* **77**, 537–549 (1994).
- Chuang, T. H., Xu, X. M., Quilliam, L. A. & Bokoch, G. M. *Biochemical J.* **303**, 761–767 (1994).
- Shinjo, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **87**, 9853–9857 (1990).
- Ridley, A. J. & Hall, A. *Cell* **70**, 389–399 (1992).
- Ridley, A. J., Paterson, H., Johnston, C., Diekmann, D. & Hall, A. *Cell* **70**, 401–410 (1992).
- Sekine, A., Fujiwara, M. & Narumiya, S. *J. Biol. Chem.* **264**, 8602–8605 (1989).
- Nishiyama, T. *et al. Molec. cell. Biol.* **14**, 2447–2456 (1994).
- Nobes, C. & Hall, A. *Curr. Opin. Genet. Dev.* **4**, 77–81 (1994).
- Cook, S. & McCormick, F. *Nature* **369**, 361–362 (1994).
- Habets, G. G. M., van der Kammen, R. A., Stam, J. C., Michiels, F. & Collard, J. G. *Oncogene* **10**, 1371–1376 (1995).
- Bokoch, G. M. *Curr. Opin. Cell Biol.* **6**, 212–218 (1994).
- Qiu, R.-G., Chen, J., Kirm, D., McCormick, F. & Symons, M. *Nature* **374**, 457–459 (1995).
- Evan, G. I. *et al. Molec. cell. Biol.* **5**, 3610–3616 (1985).
- Smith, D. B. & Johnson, K. S. *Gene* **67**, 31–40 (1988).
- Collard, J. G., Schijven, J. F. & Roos, E. *Cancer Res.* **47**, 754–759 (1987).
- Dranoff, G. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 3539–3543 (1993).
- Pear, W. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **90**, 8392–8396 (1993).

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Illumination and magnification

Product review puts image analysis on stage — there is an epifluorescence illuminator, an inverted fluorescence microscope, a high-performance digital camera and an environmental scanning electron microscope.

MELLES Griot provides a new **workstation for maximum vibration isolation**, ensuring high instrument resolution and repeatability in sensitive applications (*Reader Service No. 100*). The workstation



Vibration isolation workstation.

consists of a welded steel frame, with a self-levelling pneumatic damping system, which provides both vertical and horizontal isolation for the 60-mm thick instrument platform. A range of six sizes is available.

Data Cell offers the Mirager microscopy system, a complete **imaging workstation** for the laboratory (*Reader Service No. 101*). The system uses Optimas version 5.0 and is combined with Imaging Technology's IC-PCI frame grabber to give high-quality acquisition in conjunction with a wide range of analytical tools and application extensions. The camera's use 3-chip RGB, $1K \times 1K$ digital representation, and a low-noise monochrome digitization. There are dual monitors, an optical disk or CD-ROM storage, LibraryPro image database software and Microsoft office and network integration.

■ Fluorescent techniques

Omega Optical offers a six-page booklet that lists more than 60 **filter sets for fluorescence microscopy** (*Reader Service No. 102*). Included are the twelve Vivid multi-band sets that are said to achieve signal-to-noise levels equivalent to that of high-performance, single-band sets. These are designed for dual, triple and quadruple labelling. Information is provided on how to optimize filter sets to a given application, and on how to choose a light source or detector. Filters are mounted and aligned in filter holders compatible with all available epifluorescence microscopes.

M E Electronics has developed a range of specialized **software packages to enhance its range of scientific imaging systems** (*Reader Service No. 103*). The Fluorescence Gem uses frame-average

enhancement that allows the user to improve contrast and reduce the noise of low-intensity fluorescent images. Sequence focusing allows the user to grab a sequence of images from a video camera mounted on a microscope as the focus is altered through the sample. The image can then be reconstructed to show all planes of focus on a single output image. The colour-composite image feature is designed to allow two greyscale images of the same area showing different fluorescent stains to be combined into a single colour image.

Nikon has introduced a new **epifluorescence illuminator** that accepts up to four fluorescent filter cubes and dramatically improves brightness and contrast — important benefits for researchers working with multiple fluorochromes (*Reader Service No. 104*). The new Quadfluor epifluorescence illuminator is compatible with all Nikon Optiphot and Labphot microscopes. The new illuminator is designed to allow researchers doing fluorescence *in-situ* hybridization and other advanced fluorescence techniques to work with four or more probes without having to stop and replace filter cubes.

■ Microscopes

Leica is introducing two new **stereo-microscopes** to the market, the MS5 and the MZ6 (*Reader Service No. 105*). The MS5 has a five-step magnification changer and the MZ6 has 1:6 zoom. The ergonomic design of these new stereomicroscopes is intended to provide a fatigue-free work



Leica's MS5 and MZ6 microscopes.

environment. Comfortable handrests and control knobs are provided.

The new Olympus IX 70 and IX 50 **inverted microscopes** are said to offer important benefits for performing incident light fluorescence microscopy (*Reader Service No. 106*). Both IX inverted models have a single-reflection light path, with highly reflective mirrors and apochromatically corrected relay lenses. This is designed to produce aberration-free, ultra-bright images for fluorescence microscopy and photomicrography. The universal apochromat objective series is optimized for ultraviolet transmission in the 340-nm, near-UV range, giving it a performance that is compatible with Fura 2 ratioing and other advanced techniques. A four-cube filter turret is designed to give the IX inverted microscopes quick filter cube changeover. These scopes allow transmitted-illumination techniques such as Nomarski DIC, to be combined with reflected fluorescence.

■ Electron microscopy

Printerface is a new **microscope-to-computer interface system for scanning electron microscopes (SEMs)** that has been released by K E Developments (*Reader Service No. 107*). Available in both DOS and Window versions, it allows images to be acquired digitally into a PC directly from the SEM. Images can then be stored and archived electronically or printed via video-printers or laser printers. Printerface does not control the SEM, but locks on to the scan generator's own blanking signals; this is designed to ensure that the normal function of the microscope is not compromised. This also allows it to be compatible with most commercially available SEMs, and allows older models to benefit from the latest computer processing methods.

In order to extract maximum information from images, diffraction patterns and energy loss spectra acquired with a transmission electron microscope (TEM), Carl Zeiss has developed a new **high-performance digital camera** (*Reader Service No. 108*). The camera is operated in conjunction with the Digivision and Esivision image processing and analysis systems for the TEMs and EFTEMs (energy-filtering transmission electron microscopes) from Carl Zeiss. This design is intended to provide high sensitivity and dynamic range, uniformity of gain, a low signal-to-noise ratio, a large frame size, multispeed operation and easy handling.

The camera head is a scientific-grade, Peltier-coupled, mega-pixel charge-coupled device with an active frame size of 19×19 mm. According to the manufacturer points as small as $30 \mu\text{m}$ in diameter can be resolved by this chip.

Topometrix has released the Accurex scanning probe microscopes (SPMs) with Truemetrix closed-loop scanner linearization (*Reader Service No. 109*). This is designed to provide improved linearity and accuracy. It can handle samples up to



Topometrix's Accurex SPMs.

36 cm in diameter and up to 10 cm in thickness. Multiview dual optics allow for easy location of features of interest and guiding of the tip to the sample. The system is controlled with a Window's environment. A seven-to-one zoom with a 90° view helps the user quickly locate micrometre-sized features, and a 45° view provides the optimum perspective to guide the approach of the SPM tip to the sample safely.

A new variable vacuum scanning electron microscope (SEM) with a graphical user interface and mouse control has been launched by Hitachi Scientific Instruments (*Reader Service No. 110*). The S-3200N is designed to improve the resolution of images, increase the lifetime of the filament and make the SEM easier to use. The variable vacuum SEM allows samples to be examined at low vacuum without the need for coating. This saves on preparation time and allows 'wet' and oily samples to be examined without having to be dried out. The changeover from variable vacuum to high vacuum, or vice versa, is said to take a minute. The resolution is stated to be 3.5 nm to 5.5 nm at high and low vacuums, respectively.

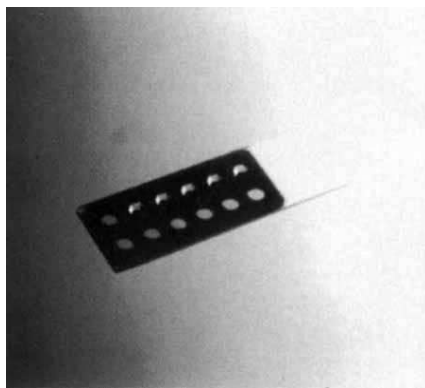
New from Electroscan is the Nikon ESEM-2700, an environmental scanning electron microscope (SEM) that utilizes a secondary electron detector (*Reader Service No. 111*). This instrument is designed to provide non-destructive testing of a wide range of materials up to 15 cm in diameter without special preparation. It features a 45° conical lens that enables the imaging of large samples at short working distances. The manufacturer states that

wet, oily or non-conductive specimens can be observed at high resolution without any preparation. Observations that are possible with this feature include the freezing and melting processes of specimens and crystallization of water-soluble crystals. Changes in state of a specimen can also be observed during heating or cooling.

Philips Optics has just released a full colour brochure outlining the features and benefits of the XL series of scanning electron microscopes (*Reader Service No. 112*). The series has incorporated computer technology using a Windows platform to provide ease of operation. A choice of chambers, stages and electron guns can be used. Standard system software will operate a variety of detectors that can be integrated. The XL series is capable of digital and analog image production, as well as X-ray analysis and full colour picture output.

Electron Microscopy Sciences has released its 1995 product catalogue (*Reader Service No. 113*). It includes 400 pages of up-to-date chemicals, supplies and equipment for the fields of microscopy, biology and histology. There are 19 chapters covering chemicals, embedding kits, grids, staining supplies, calibration standards, microanalysis element standards, specimen preparation supplies, materials science and metrology supplies.

Cel-Line's custom heavy Teflon-coated (HTC) slides are available on MicroPure optical-quality glass in seven standard colours (*Reader Service No. 114*). Acetone-proof, chemical-resistant and autoclavable, HTC colour slides are designed to be



Cel-Line's heavy Teflon-coated slides.

extremely hydrophobic, preventing cross contamination and increasing microlitre capacities. The opaque coating is designed to eliminate glare. Cel-Line colour slides are available in standard 1 to 80 well configurations. □

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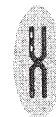
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